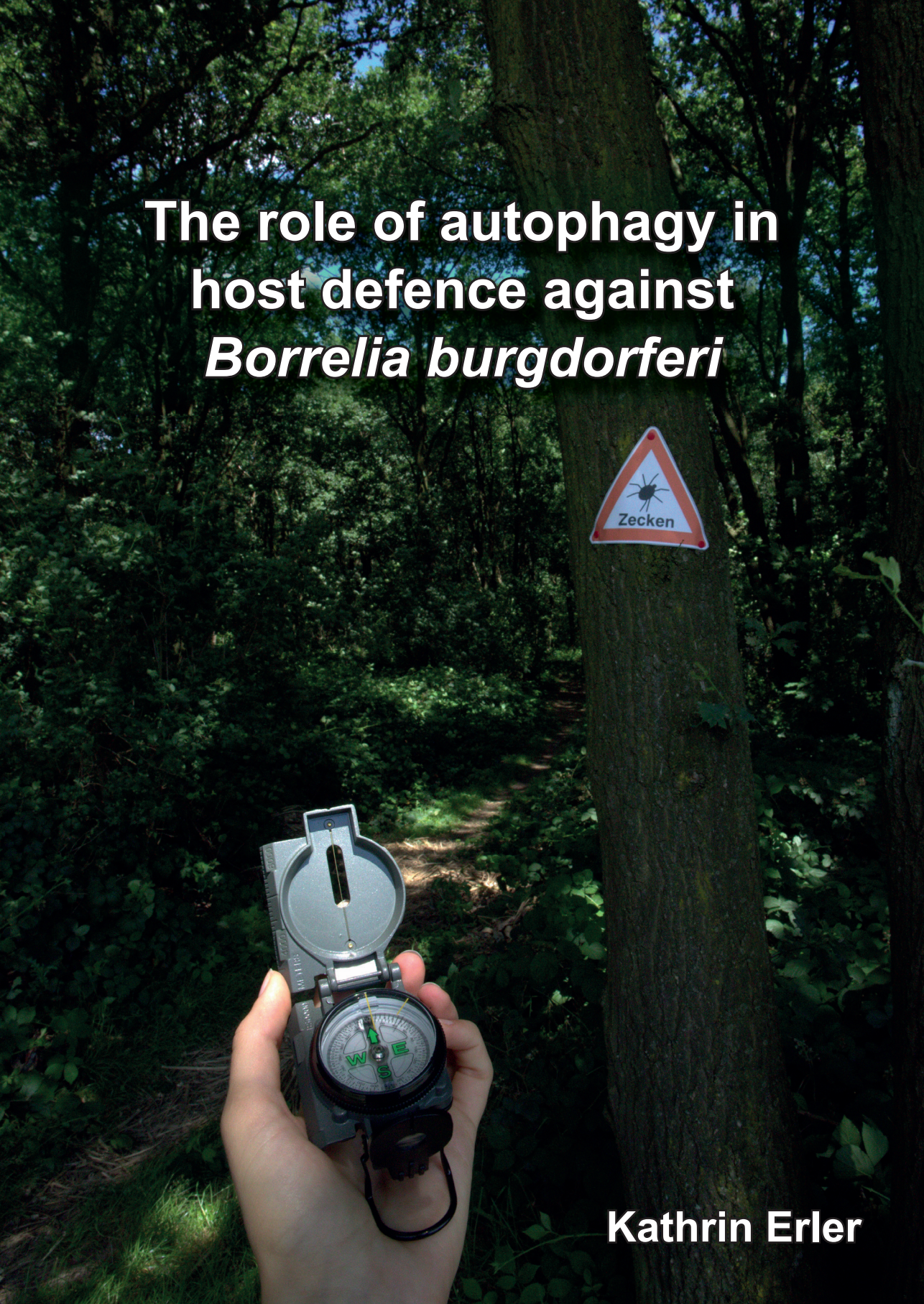
The role of autophagy in host defence against *Borrelia burgdorferi*



Kathrin Erler

Colofon
The research presented in this thesis was performed at the Department of Internal Medicine, Radboudumc, Nijmegen, The Netherlands

Printed by
Print.Point GbR

Cover design by
Kathrin Erler

The role of autophagy in host defence against *Borrelia burgdorferi*

Proefschrift

ter verkrijging van de graad van doctor
aan de Radboud Universiteit Nijmegen
op gezag van de rector magnificus prof. dr. J.H.J.M. van Krieken,
volgens besluit van het college van decanen
in het openbaar te verdedigen op donderdag 22 december 2016
om 10.30 uur precies

door

Kathrin Erler

geboren op 23 oktober 1986
te Kevelaer, Duitsland

Promotoren
Prof. dr. L.A.B. Joosten
Prof. dr. M.G. Netea

Copromoter
Dr. M. Doppenberg-Oosting

Manuscriptcommissie
Prof. dr. R.P. Pickkers
Prof. dr. W.B. van den Berg
Prof. dr. T. van der Poll (AMC)

Table of contents

Chapter 1

Introduction and outline of the thesis

Adapted from: Innate immunity networks during infection with *Borrelia burgdorferi*. Crit Rev Microbiol. 2016 Mar;42(2):233-44.

Marije Oosting, Kathrin Buffen, Jos W.M. van der Meer, Mihai G. Netea, Leo A.B. Joosten

General introduction

Lyme disease, caused by spirochetes of the *Borrelia burgdorferi* sensu lato complex, is the most common vector-born disease in the northern hemisphere. The Centers for Disease Control and Prevention (CDC) reported over 300,000 cases of confirmed Lyme disease patients in the United States of America in 2013, whereas already 22,000 cases of erythema migrans (EM) were reported in the Netherlands in 2010.
Since the original description of the disease by Steere and co-workers in 1975 [1] and the discovery of the causative pathogen by Burgdorfer in 1982 [2], our knowledge of the etiology and pathogenesis of Lyme disease has increased tremendously. The first most-common sign of infection manifest as early localized skin inflammation (EM), frequently accompanied by flu-like symptoms [3]. If treated correctly, the prognosis for these patient is excellent; however, if untreated, hematogenous dissemination of spirochetes may give rise to a wide range of clinical manifestations, involving the central nervous system (mainly *caused by Borrelia garinii*), the skin (*Borrelia afzelii*) or the joints (*Borrelia burgdorferi*). Although clinicians still have difficulties making the diagnosis, signs and symptoms of the infection are nowadays better recognized and this results in early antibiotic treatment in many patients. In approximately 10% of the patients symptoms may persist, despite antibiotic treatment [4]. To what extent the prolonged illness is due to persistent infection, persistent non-infectious inflammation or neither is controversial. Thus more insight into the pathogenesis of Lyme disease and its sequelae will help in the management of patients to develop novel treatment modalities in the future.

Borrelia characteristics and early host defense

Borrelia burgdorferi, B. afzelii, and *B. garinii* are Gram-negative bacteria. The spirochetal flat-waved body of *Borrelia* has a typical coiled spiral shape, with lengths ranging from 3 to 500 µM, and a diameter ranging from 0.09 to 0.75 µM [5]. This variation in diameter might be dependent on the environment where the spirochetes were originated, but not crucial for their pathogenic behavior.
Ticks within the *Ixodidae* family inject the disease-causing bacteria into the host's bloodstream. Since *Borrelia* is sensitive to proteases that are released by the host [6-8] it expresses a variety of proteins on their outer surface that modulates the defense system of the host. One family of proteins expressed by Borrelia belongs to the outer surface proteins (OSP) family, of which OspA and OspC are best known. OspC is expressed during early stages of infection, and has recently been described to bind host plasminogen and thereby support dissemination of the *Borrelia* bacterium [9]. When OspC is bound to plasminogen, it is more difficult for the host defense system to recognize the pathogen.

Recognition of *Borrelia burgdorferi*

The immune system of the host is able to react to pathogenic bacteria in a short timeframe with a wide area of different immune cells. Already in 1984 it was described that *Borrelia* could be recognized and phagocytosed by neutrophils and macrophages

[10]. In conjunction with phagocytosis, neutrophils are capable of eliminating *Borrelia* by oxidative burst, and release of lysosomal hydrolytic enzymes. Chemoattractants for polymorphonuclear (PMN) leukocytes have been detected in human synovial fluids from *Borrelia*-infected knee joints [11].
Cells of the immune system need to be activated before cytokines or chemokines can be produced. These activation mechanisms can be induced after the recognition of highly specific microbial ligands, so-called pathogen-associated molecular patterns (PAMPs), by pattern recognition receptors (PRRs). Several families of PRRs have been described, including Toll-like receptors (TLRs), Nod-like receptors (NLRs), C-type lectins (CTLs), and RigI-helicases. Numerous in vitro studies indicate the importance of lipid-modified proteins in the pathogenesis of the infection [12-14]. The immune system recognizes these lipoproteins by different pattern recognition receptors (PRRs), of which Toll-like receptor 2 (TLR2) and Nucleotide-binding oligomerization domain-containing protein 2 (NOD2) are suggested to be the most important during the pathogenesis of *Borrelia burgdorferi* infection (Figure 1) [15, 16].

Role of extracellular receptors for *Borrelia* recognition - TLR1, TLR2, and TLR6
The TLR-family comprises 11 human and 13 murine receptors. TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10 are mainly found on the surface of cells whereas TLR3, TLR7, TLR8, and TLR9 are located intracellularly. TLR11 is nonfunctional in humans due to an early stop codon in the coding gene [17]. Each of the TLRs recognizes a specific set of microbial ligands.
By recognizing lipopeptides (e.g. OspA) from *Borrelia* species [18, 19], TLR2 represents a key recognition receptor present on immune cells [20]. Blockade of TLR2 leads to an abrogated immune response induced by *Borrelia* exposure in human primary cells [21]. Furthermore, variations in the human TLR2 gene appear to result in different clinical outcomes in Lyme disease [22].

TLR2 forms either homodimers, or heterodimers with TLR1 or TLR6. By interacting with co-receptors, TLR2 is able to recognize a broad range of microbial ligands, including peptidoglycans, lipopeptides and fungal polysaccharides [23]. Until recently, it was unclear whether *Borrelia* was recognized by either TLR1/2 or TLR2/6 heterodimers. By using RNA silencing approaches it was shown that TLR1, rather than TLR6, is important for the recognition of *Borrelia* by the host defense system [15, 24-26]. There is additional evidence that TLR1 and TLR2 are important in a murine model of Lyme disease. Wooten et al. demonstrated that higher spirochetal numbers were found in joints of TLR2-deficient mice 8 weeks after intradermal infection with live B. *burgdorferi* [22]. Higher amounts of Borrelia have also been observed in skin and bladder of TLR1/2 deficient mice after transmission of *Borrelia* via tick bites [27]. In addition, *Borrelia*-induced Lyme arthritis was significantly enhanced in mice missing TLR1 or TLR2, as demonstrated by increased local cell influx after OspA immunization or intradermal *Borrelia* injection [28, 29]. TLR1/2 heterodimers are therefore crucial for the recognition and for controlling the outgrowth of *Borrelia* in vivo, as well as for Borrelia-induced inflammation.

Role of intracellular receptors for *Borrelia* recognition – NOD2

NOD-like receptors (NLR) family members are a separate class of intracellular PRR. The major pattern recognition receptors within this family are the Nucleotide Oligomerization Domain (NOD) members 1 and 2, as well as the NLRP receptors that are involved in inflammasome activation. NOD1 is activated by muramyltripeptide (MTP), derived from Gram-negative bacteria, while NOD2 is activated by muramyldipeptide (MDP) from Gram-positive bacteria. After activation of either NOD1 or NOD2 by these bacterial moieties, activation of the adaptor molecule RICK finally leads to transcription of NFκB and production of pro- and anti-inflammatory cytokines (Figure 1).

It was long assumed that only the proteins on the outer surface of *Borrelia* cell wall are responsible for the induction of inflammatory mediators [22]. Oosting et al, however, demonstrated that although *Borrelia* is a Gram-negative organism, NOD2 is involved in *Borrelia*-induced cytokine responses of human PBMCs [21]. Individuals bearing a genetic polymorphism leading to a dysfunctional NOD2 molecule display a decreased cytokine production. In line with these data, murine BMDCs lacking NOD2 also produced less cytokines after live B. *burgdorferi* exposure [30]. In addition, the production of cytokines by synovial tissue explants of NOD2-deficient mice that were injected intraarticularly with live *B. burgdorferi* was reduced [31]. However, the inhibition of cytokine production was not complete, indicating that NOD2-independent signaling pathways are relevant. The NOD2-deficient BMDMs exposed to *Borrelia* also exhibit a downregulated type I interferon response [30], but the role of NOD2 for the type I interferon production is largely redundant [32].

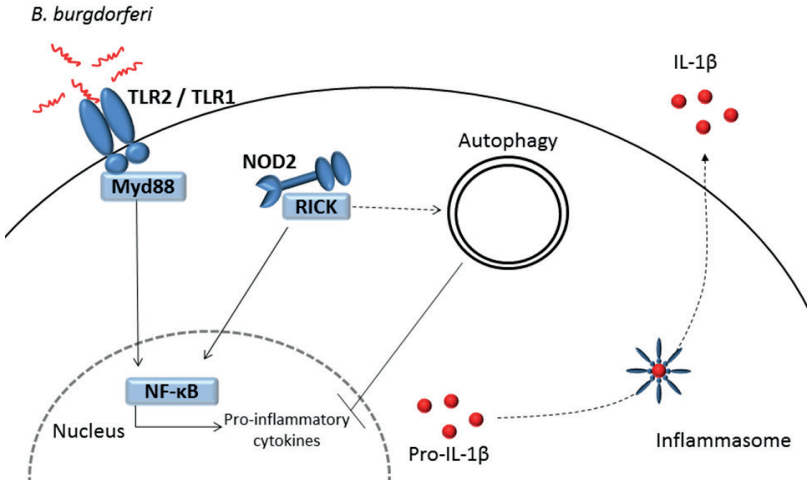


Figure 1. Schematic overview of *Borrelia*-induced autophagy and its effect on cytokines. *Borrelia* bacteria are recognized through specific pattern recognition receptors, including Toll-like receptor 1/2 and NOD2. After activation, NF-κB is transcribed and cytokines, such as Il-1β are produced and secreted after activation by the inflammasome. In addition, *B. burgdorferi* induces autophagy, which regulates the production of inflammatory cytokines.

Autophagy

In addition to the induction of intracellular signals leading to the production of cytokines, engagement of PRRs such as NOD2 activates autophagy, a process in which damaged organelles or long-lived proteins are degraded [33-36]. Autophagy involves the sequestration of dysfunctional proteins in a double-layered membrane called autophagosome, which is formed by the elongation of small membrane structures. The formation of the isolation membrane is initiated by several kinases as ULK1 and ULK2 (which are mammalian homologues of the yeast autophagy-related protein 1 – ATG1) and the activity of type III phosphatidylinositol 3-kinase (PI3K) which forms a complex with beclin 1 (yeast ATG6) [37]. Further elongation and autophagosome completion requires different complexes of autophagy proteins as the ATG5-ATG12-ATG16L1 complex and the PE-conjugated microtubule-associated protein 1 light chain 3 (LC3) [38]. The delivery of dysfunctional proteins to the autophagosomes is regulated by autophagic adaptors such as p62 which can bind to the intracellular target as well as to LC3, which associates to the autophagosome after being processed [39]. Autophagosomes mature through fusion with lysosomes, leading to the breakdown of the protein content [40].

***Borrelia*-induced cytokines**

Proinflammatory cytokines play an important role in the pathogenesis of Lyme disease. Early during the infection, monocytes and macrophages that encounter live *Borrelia*, produce several pro-inflammatory cytokines, such as IL-1β, IL-6, IL-8 and TNF-α [25, 41-44]. The mRNA coding for interleukin-1β can be detected as early as 1h after exposure to *Borrelia* in human cells. These cytokines activate phagocytes for the killing of the pathogens after recognition, and in addition attract other immune cells to the site of infection. The monocyte-derived cytokines also induce T lymphocyte polarization leading to production of interferon-γ (by T helper 1 lymphocytes) and IL-17 (by T helper 17 cells). In skin biopsies taken from patients presenting with an erythema migrans, high levels of pro-inflammatory cytokines including IFN-γ could be detected [45].

The role of IL-1β

IL-1β is one of the cytokines that is produced in high concentrations by both murine and human monocytes/macrophages after exposure to *Borrelia* species (Figure 2) [21, 43, 46, 47]. Of interest IL-1β was detected in synovial fluid of Lyme arthritis patients at early time points [48]. Peptidoglycan from the cell wall of *Borrelia* is the main inducer of the production of IL-1β by murine macrophages [49]. Mice susceptible for the development of Lyme disease (C3H/HeN strain) were found to respond with high cytokine production when challenged with *Borrelia* spirochetes [50]. The development of murine Lyme arthritis is critically dependent on IL-1β as demonstrated in IL-1R deficient mice [31]. In patients with Lyme arthritis, whose arthritis was reactivated after antibiotic treatment it was shown that IL-1β concentrations were higher in synovial fluid and tissue than in patients that recovered from the antibiotic treatment [48]. Still, the exact role of IL-1β in the development of persistent Lyme disease is not fully understood. It is likely that at least part of the

pathogenic role of IL-1β in Lyme disease is mediated via the induction of a proper IL-17/Th17 response against *Borrelia* spirochetes, with subsequent production of IL-22 [46]. IL-17 is a cytokine that plays an important role in the Th17-mediated responses and can amplify immune activation upon microbial recognition [46].

The role of IL-23, IL-17, IL-22, and IFN-γ

IL-23, IL-17, IL-22 and IFN-γ are produced after exposure of *Borrelia* to human peripheral blood mononuclear cells [25, 46, 51]. Production of IL-17 and IL-22 is under control of IL-1β, and the naturally-occurring IL-1 receptor antagonist (IL-1Ra) inhibits the Th17-derived cytokine response [46]. In line with these data, IL-1β-deficient mice have a defective Th17 response [44]. IL-17 is important in the development of the *Borrelia*-antigen induced arthritis model as shown by experiments of Burchill et al, in which IL-17 blockade resulted in diminished joint inflammation [52, 53]. IFN-γ and IL-22 were detected in skin biopsies from individuals with erythema migrans [54], and IL-17 production was found in synovial cells from Lyme arthritis patients [55]. Patients with proven late neuroborreliosis exhibited higher IL-17 concentrations than patients with earlier stages of neuroborreliosis [56].

IL-23 signaling is needed for optimal Th17 development, and PBMCs isolated from healthy subjects bearing a SNP in IL-23R showed a significantly decreased IL-17 production when exposed to Borrelia. The presence of this IL-23R SNP was associated with a trend towards a lower frequency of chronic clinical symptoms in a cohort of Lyme patients [51].

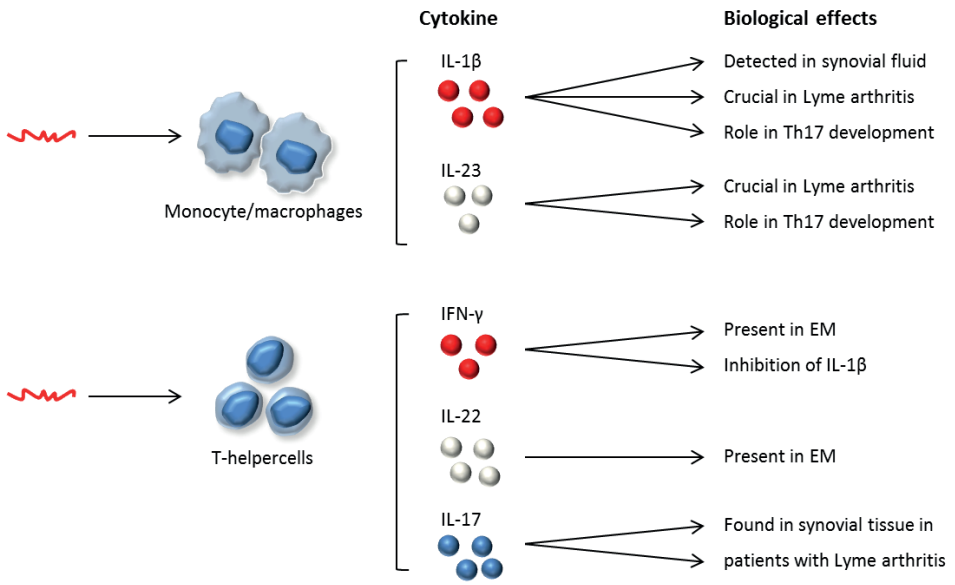


Figure 2. *Borrelia*-induced cytokines. After recognition of *Borrelia* by host immune cells, induction of early (upper part) cytokines is induced. Each cytokine has a different function in the host immune response against this bacterium.

Autophagy and Lyme disease

The link between autophagy and the innate defense mechanism has been made in several studies describing the connection between dysfunctional autophagy and autoinflammatory diseases [57-60]. It has been shown that inhibition of autophagy by chemical inhibitors of PI3 kinases leads to an enhanced production of extracellular IL-1β after stimulation with bacterial wall components such as LPS [61]. This observation, next to the fact that *B. burgdorferi* is thought to be recognized by the autophagy-inducing receptor NOD2, led to the investigation of the role of autophagy during infection with *B. burgdorferi*. It was shown that autophagy-blocked PBMCs stimulated with the *Borrelia*-spirochete secreted higher levels of IL-1β and IL-6 protein. In addition, the mRNA synthesis of IL-1β and IL-6 was strongly increased as well, indicating that the inhibition of autophagy modulates IL-1β and IL-6 production at the transcriptional level (Figure 1) [62].

Furthermore, it has been shown that autophagy modulates the production of T-cell derived cytokines as IL-17, IL-22 and IFN-γ [63, 64], all cytokines known to be produced also after stimulation with *B. burgdorferi* [65, 66]. Since higher levels of IL-17 have been associated to increased joint damage in rheumatoid arthritis patients [67] and elevated IL-17 levels have been found in patients with confirmed neuroborreliosis [68], the role of autophagy on the production of T cell-derived cytokines in response to *B. burgdorferi* has been examined. It was shown that autophagy-blocked PBMCs stimulated with the *Borrelia*-spirochete secreted higher levels of IL-17, IL-22 and IFN-γ protein [69], demonstrating a regulatory link between autophagy and T-cell cytokine production in response to *B. burgdorferi* stimulation, which was dependent on IL-1 but not on IL-23 secretion.

Outline of the thesis

In recent years, the knowledge of the *Borrelia* spirochete and Lyme disease has greatly increased. Considerable insight has been gained into how the encounter between the microorganism and the host leads to disease. Nevertheless, many aspects of the disease caused by *Borrelia* spirochetes remain unexplained, and the nature of the immune response against this bacterium is only partly understood. The aim of this thesis is to study the role of autophagy during the immune response against *B. burgdorferi* and to identify potential novel therapeutic targets for the treatment of Lyme disease.

In **Chapter 2** the signaling pathways – TLR2-Myd88 and NOD2-RICK – involved in the recognition of *B. burgdorferi* by immune cells and their effect on the induction of cytokines will be elucidated. The recognition of *Borrelia* results in the secretion of the pro-inflammatory cytokine IL-1β which is known to be involved in the pathogenesis of Lyme disease [70-72]. IL-1β production requires two important steps: transcription of mRNA resulting in the production of proIL-1β protein, and cleavage of the immature precursor into mature bioactive IL-1β by the inflammasome-enzyme caspase-1 [73]. Therefore, the role of the NLRP3 inflammasome for *Borrelia*-induced Lyme arthritis will be explored. Several studies linked the activation of NOD2 to autophagy [74]. Autophagy is an intracellular mechanism that regulates degradation of old or damaged cell organelles without any

harmful effect on the cell itself. When autophagy is hampered or not functional due to a genetic defect, IL-1β production is strongly elevated after exposure to *B. burgdorferi* as shown in **Chapter 3**. IL-1β has a broad range of functions as mediating inflammation and providing protective immunity to infectious diseases. In synergy with IL-23, it induces the production of IL-17 which has been associated with increased joint damage in patients with rheumatoid arthritis [67]. In **Chapter 4** the role of autophagy in a knockout mouse model of Lyme arthritis will be illustrated, followed by the effect of autophagy on *Borrelia*-induced adaptive cytokines. The autophagy machinery depends on specialized autophagy-related proteins (ATGs). To identify the key players of autophagy, which are modulated by stimulation with the *Borrelia* spirochete, transcriptional analysis and functional genomics will be performed in **Chapter 5**.

In recent years, emerging evidence has shown that after infection or vaccination, innate immune cells display long-term changes in their functional programs. These changes lead to increased production of inflammatory mediators and enhanced capacity to eliminate infection. In **Chapter 6** the role of autophagy in trained immunity will be investigated, demonstrating a possible protective role for future infections with *B. burgdorferi.*

A summary of the new findings and conclusions of this thesis are presented in **Chapter 7**.

References

1. Steere, A.C., et al., *Lyme arthritis: an epidemic of oligoarticular arthritis in children and adults in three connecticut communities.* Arthritis Rheum, 1977. **20**(1): p. 7-17.

2. Burgdorfer, W., et al., *Lyme disease-a tick-borne spirochetosis?* Science, 1982. **216**(4552): p. 1317-9.

3. Wormser, G.P., et al., *The clinical assessment, treatment, and prevention of lyme disease, human granulocytic anaplasmosis, and babesiosis: clinical practice guidelines by the Infectious Diseases Society of America.* Clin Infect Dis, 2006. **43**(9): p. 1089-134.

4. Steere, A.C., J. Coburn, and L. Glickstein, *The emergence of Lyme disease.* J Clin Invest, 2004. **113**(8): p. 1093-101.

5. C.M. Olson, E.F., Juan Anguita, *Clinical Immunology*. Vol. 4th. 2013. 338-345.

6. Guyard, C., et al., *Relapsing fever spirochaetes produce a serine protease that provides resistance to oxidative stress and killing by neutrophils.* Mol.Microbiol., 2006. **60**(3): p. 710-722.

7. Kumru, O.S., et al., *Specificity and role of the Borrelia burgdorferi CtpA protease in outer membrane protein processing.* J.Bacteriol., 2011. **193**(20): p. 5759-5765.

8. Fraser, C.M., et al., *Genomic sequence of a Lyme disease spirochaete, Borrelia burgdorferi.* Nature, 1997. **390**(6660): p. 580-586.

9. Onder, O., et al., *OspC is a potent plasminogen-receptor on the surface of Borrelia burgdorferi.* J.Biol.Chem., 2012.

10. Benach, J.L., et al., *Interactions of phagocytes with the Lyme disease spirochete: role of the Fc receptor.* J Infect Dis, 1984. **150**(4): p. 497-507.

11. Georgilis, K., et al., *Neutrophil chemotactic factors in synovial fluids of patients with Lyme disease.* Arthritis Rheum, 1991. **34**(6): p. 770-5.

12. Vincent, M.S., et al., *Lyme arthritis synovial gamma delta T cells respond to Borrelia burgdorferi lipoproteins and lipidated hexapeptides.* J Immunol, 1998. **161**(10): p. 5762-71.

13. Hefty, P.S., et al., *Regulation of OspE-related, OspF-related, and Elp lipoproteins of Borrelia burgdorferi strain 297 by mammalian host-specific signals.* Infect Immun, 2001. **69**(6): p. 3618-27.

14. Norgard, M.V., et al., *Activation of human monocytic cells by Treponema pallidum and Borrelia burgdorferi lipoproteins and synthetic lipopeptides proceeds via a pathway distinct from that of lipopolysaccharide but involves the transcriptional activator NF-kappa B.* Infect Immun, 1996. **64**(9): p. 3845-52.

15. Oosting, M., et al., *TLR1/TLR2 heterodimers play an important role in the recognition of Borrelia spirochetes.* PLoS One, 2011. **6**(10): p. e25998.

16. Oosting, M., et al., *Recognition of Borrelia burgdorferi by NOD2 is central for the induction of an inflammatory reaction.* J Infect Dis, 2010. **201**(12): p. 1849-58.

17. Lauw, F.N., D.R. Caffrey, and D.T. Golenbock, *Of mice and man: TLR11 (finally) finds profilin.* Trends Immunol., 2005. **26**(10): p. 509-511.

18. Akira, S. and K. Takeda, *Toll-like receptor signalling.* Nat.Rev.Immunol., 2004. **4**(7): p. 499-511.

19. Lien, E., et al., *Toll-like receptor 2 functions as a pattern recognition receptor for diverse bacterial products.* J.Biol.Chem., 1999. **274**(47): p. 33419-33425.

20. Hirschfeld, M., et al., *Cutting edge: repurification of lipopolysaccharide eliminates signaling through both human and murine toll-like receptor 2.* J.Immunol., 2000. **165**(2): p. 618-622.

21. Oosting, M., et al., *Recognition of Borrelia burgdorferi by NOD2 is central for the induction of an inflammatory reaction.* J.Infect.

Dis., 2010. **201**(12): p. 1849-1858.
22. Wooten, R.M., et al., *Toll-like receptor 2 is required for innate, but not acquired, host defense to Borrelia burgdorferi.* J.Immunol., 2002. **168**(1): p. 348-355.
23. Oliveira-Nascimento, L., P. Massari, and L.M. Wetzler, *The Role of TLR2 in Infection and Immunity.* Front Immunol, 2012. **3**: p. 79.
24. Bulut, Y., et al., *Cooperation of Toll-like receptor 2 and 6 for cellular activation by soluble tuberculosis factor and Borrelia burgdorferi outer surface protein A lipoprotein: role of Toll-interacting protein and IL-1 receptor signaling molecules in Toll-like receptor 2 signaling.* J.Immunol., 2001. **167**(2): p. 987-994.
25. Salazar, J.C., et al., *Activation of human monocytes by live Borrelia burgdorferi generates TLR2-dependent and -independent responses which include induction of IFN-beta.* PLoS.Pathog., 2009. **5**(5): p. e1000444.
26. Takeuchi, O., et al., *Discrimination of bacterial lipoproteins by Toll-like receptor 6.* Int. Immunol., 2001. **13**(7): p. 933-940.
27. Fikrig, E., et al., *Toll-like receptors 1 and 2 heterodimers alter Borrelia burgdorferi gene expression in mice and ticks.* J.Infect.Dis., 2009. **200**(8): p. 1331-1340.
28. Alexopoulou, L., et al., *Hyporesponsiveness to vaccination with Borrelia burgdorferi OspA in humans and in.* Nat.Med., 2002. **8**(8): p. 878-884.
29. Wang, X., et al., *T cell infiltration is associated with increased Lyme arthritis in TLR2-/- mice.* FEMS Immunol.Med.Microbiol., 2008. **52**(1): p. 124-133.
30. Petnicki-Ocwieja, T., et al., *Nod2 suppresses Borrelia burgdorferi mediated murine Lyme arthritis and carditis through the induction of tolerance.* PLoS.One., 2011. **6**(2): p. e17414.
31. Oosting, M., et al., *Murine Borrelia arthritis is highly dependent on ASC and caspase-1, but independent of NLRP3.* Arthritis Res Ther, 2012. **14**(6): p. R247.
32. Miller, J.C., et al., *The Lyme disease spirochete Borrelia burgdorferi utilizes multiple ligands, including RNA, for interferon regulatory factor 3-dependent induction of type I interferon-responsive genes.* Infect.Immun., 2010. **78**(7): p. 3144-3153.
33. Cooney, R., et al., *NOD2 stimulation induces autophagy in dendritic cells influencing bacterial handling and antigen presentation.* Nat Med, 2010. **16**(1): p. 90-7.
34. Plantinga, T.S., L.A. Joosten, and M.G. Netea, *ATG16L1 polymorphisms are associated with NOD2-induced hyperinflammation.* Autophagy, 2011. **7**(9): p. 1074-5.
35. Netea, M.G. and L.A. Joosten, *A NOD for autophagy.* Nat Med, 2010. **16**(1): p. 28-30.
36. Anand, P.K., et al., *TLR2 and RIP2 pathways mediate autophagy of Listeria monocytogenes via extracellular signal-regulated kinase (ERK) activation.* J Biol Chem, 2011. **286**(50): p. 42981-91.
37. Deretic, V., T. Saitoh, and S. Akira, *Autophagy in infection, inflammation and immunity.* Nat Rev Immunol, 2013. **13**(10): p. 722-37.
38. Oh, J.E. and H.K. Lee, *Modulation of pathogen recognition by autophagy.* Front Immunol, 2012. **3**: p. 44.
39. Ponpuak, M., et al., *Delivery of Cytosolic Components by Autophagic Adaptor Protein p62 Endows Autophagosomes with Unique Antimicrobial Properties.* Immunity, 2010. **32**(3): p. 329-341.
40. Kundu, M. and C.B. Thompson, *Autophagy: Basic principles and relevance to disease.* Annual Review of Pathology-Mechanisms of Disease, 2008. **3**: p. 427-455.
41. Petzke, M.M., et al., *Recognition of Borrelia burgdorferi, the Lyme disease spirochete, by TLR7 and TLR9 induces a type I IFN response by human immune cells.* J.Immunol., 2009. **183**(8): p. 5279-5292.
42. Miller, L.C., et al., *Live Borrelia burgdorferi preferentially activate interleukin-1 beta gene expression and protein synthesis over the interleukin-1 receptor antagonist.* J.Clin.

Invest, 1992. **90**(3): p. 906-912.

43. Porat, R., et al., *Interleukin-1 (IL-1) receptor blockade reduces endotoxin and Borrelia burgdorferi-stimulated IL-8 synthesis in human mononuclear cells.* FASEB J., 1992. **6**(7): p. 2482-2486.

44. Oosting, M., et al., *Borrelia species induce inflammasome activation and IL-17 production through a caspase-1-dependent mechanism.* Eur.J.Immunol., 2011. **41**(1): p. 172-181.

45. Mullegger, R.R., et al., *Chemokine signatures in the skin disorders of Lyme borreliosis in Europe: predominance of CXCL9 and CXCL10 in erythema migrans and acrodermatitis and CXCL13 in lymphocytoma.* Infect.Immun., 2007. **75**(9): p. 4621-4628.

46. Bachmann, M., et al., *Early production of IL-22 but not IL-17 by peripheral blood mononuclear cells exposed to live Borrelia burgdorferi: the role of monocytes and interleukin-1.* PLoS. Pathog., 2010. **6**(10): p. e1001144.

47. Beck, G., et al., *A role for interleukin-1 in the pathogenesis of Lyme disease.* Zentralbl. Bakteriol.Mikrobiol.Hyg.A, 1986. **263**(1-2): p. 133-136.

48. Shin, J.J., L.J. Glickstein, and A.C. Steere, *High levels of inflammatory chemokines and cytokines in joint fluid and synovial tissue throughout the course of antibiotic-refractory lyme arthritis.* Arthritis Rheum., 2007. **56**(4): p. 1325-1335.

49. Beck, G., J.L. Benach, and G.S. Habicht, *Isolation, preliminary chemical characterization, and biological activity of Borrelia burgdorferi peptidoglycan.* Biochem. Biophys.Res.Commun., 1990. **167**(1): p. 89-95.

50. Isogai, H., et al., *Levels of endogenous interleukin-1, interleukin-6, and tumor necrosis factor in congenic mice infected with Borrelia garinii.* Microbiol.Immunol., 1997. **41**(5): p. 427-430.

51. Oosting, M., et al., *Role of interleukin-23 (IL-23) receptor signaling for IL-17 responses in human Lyme disease.* Infect.Immun., 2011. **79**(11): p. 4681-4687.

52. Burchill, M.A., et al., *Inhibition of interleukin-17 prevents the development of arthritis in vaccinated mice challenged with Borrelia burgdorferi.* Infect.Immun., 2003. **71**(6): p. 3437-3442.

53. Kuo, J., et al., *Interleukin-35 enhances Lyme arthritis in Borrelia-vaccinated and -infected mice.* Clin.Vaccine Immunol., 2011. **18**(7): p. 1125-1132.

54. Jones, K.L., et al., *Higher mRNA levels of chemokines and cytokines associated with macrophage activation in erythema migrans skin lesions in patients from the United States than in patients from Austria with Lyme borreliosis.* Clin.Infect.Dis., 2008. **46**(1): p. 85-92.

55. Codolo, G., et al., *Borrelia burgdorferi NapA-driven Th17 cell inflammation in lyme arthritis.* Arthritis Rheum., 2008. **58**(11): p. 3609-3617.

56. Henningsson, A.J., et al., *Indications of Th1 and Th17 responses in cerebrospinal fluid from patients with Lyme neuroborreliosis: a large retrospective study.* J.Neuroinflammation., 2011. **8**: p. 36.

57. Marquez, A., et al., *Role of ATG16L1 Thr300Ala Polymorphism in Inflammatory Bowel Disease: A Study in the Spanish Population and a Meta-analysis.* Inflammatory Bowel Diseases, 2009. **15**(11): p. 1697-1704.

58. Zhang, H.F., et al., *ATG16L1 T300A polymorphism and Crohn's disease susceptibility: evidence from 13,022 cases and 17,532 controls.* Human Genetics, 2009. **125**(5-6): p. 627-631.

59. Plantinga, T.S., et al., *Modulation of inflammation by autophagy: consequences for Crohn's disease.* Curr Opin Pharmacol, 2012.

60. Plantinga, T.S., et al., *Crohn's disease-associated ATG16L1 polymorphism modulates pro-inflammatory cytokine responses selectively upon activation of NOD2.* Gut, 2011. **60**(9): p. 1229-35.

61. Crisan, T.O., et al., *Inflammasome-independent modulation of cytokine response*

by autophagy in human cells. PLoS One, 2011. **6**(4): p. e18666.

62. Buffen, K., et al., *Autophagy modulates Borrelia burgdorferi-induced production of interleukin-1beta (IL-1beta).* J Biol Chem, 2013. **288**(12): p. 8658-66.

63. Said, A., et al., *Chloroquine promotes IL-17 production by CD4+ T cells via p38-dependent IL-23 release by monocyte-derived Langerhans-like cells.* J Immunol, 2014. **193**(12): p. 6135-43.

64. Peral de Castro, C., et al., *Autophagy regulates IL-23 secretion and innate T cell responses through effects on IL-1 secretion.* J Immunol, 2012. **189**(8): p. 4144-53.

65. Oosting, M., et al., *Borrelia species induce inflammasome activation and IL-17 production through a caspase-1-dependent mechanism.* Eur J Immunol, 2011. **41**(1): p. 172-81.

66. Bachmann, M., et al., *Early production of IL-22 but not IL-17 by peripheral blood mononuclear cells exposed to live Borrelia burgdorferi: the role of monocytes and interleukin-1.* PLoS Pathog, 2010. **6**(10): p. e1001144.

67. Geboes, L., et al., *Proinflammatory role of the Th17 cytokine interleukin-22 in collagen-induced arthritis in C57BL/6 mice.* Arthritis Rheum, 2009. **60**(2): p. 390-5.

68. Henningsson, A.J., et al., *Indications of Th1 and Th17 responses in cerebrospinal fluid from patients with Lyme neuroborreliosis: a large retrospective study.* J Neuroinflammation, 2011. **8**: p. 36.

69. Buffen, K., et al., *Autophagy suppresses host adaptive immune responses toward Borrelia burgdorferi.* J Leukoc Biol, 2016.

70. Liu, N., et al., *The caspase 1 inflammasome is not required for control of murine Lyme borreliosis.* Infect Immun, 2009. **77**(8): p. 3320-7.

71. Petzke, M.M., et al., *Recognition of Borrelia burgdorferi, the Lyme disease spirochete, by TLR7 and TLR9 induces a type I IFN response by human immune cells.* J Immunol, 2009. **183**(8): p. 5279-92.

72. Pietruczuk, A., et al., *[Role of interleukin-18, interleukin-1beta and its soluble receptor (sIL-1RII) in early and late Lyme borreliosis].* Pol Merkur Lekarski, 2004. **17**(101): p. 446-50.

73. Dinarello, C.A., *Immunological and Inflammatory Functions of the Interleukin-1 Family.* Annual Review of Immunology, 2009. **27**: p. 519-550.

74. Homer, C.R., et al., *A dual role for receptor-interacting protein kinase 2 (RIP2) kinase activity in nucleotide-binding oligomerization domain 2 (NOD2)-dependent autophagy.* J Biol Chem, 2012. **287**(30): p. 25565-76.

Chapter 2

Murine *Borrelia* arthritis is highly dependent on ASC and caspase-1, but independent of NLRP3.

Arthritis Res Ther. 2012 Nov 13;14(6): R247

Marije Oosting, Kathrin Buffen, Subbarao R.K. Malireddi, Patrick Sturm, Ineke Verschueren, Marije I. Koenders, Frank L. van de Veerdonk, Jos W.M. van der Meer, Mihai G. Netea, Thirumala-Devi Kanneganti, and Leo A.B. Joosten

Abstract

The protein platform called the Nod-Like-Receptor-family member (NLRP)-3 inflammasome needs to be activated to process intracellular caspase-1. Active caspase-1 is able to cleave pro-Interleukin (IL)-1β, resulting in bioactive IL-1β. IL-1β is a potent pro-inflammatory cytokine, and thought to play a key role in the pathogenesis of Lyme arthritis, a common manifestation of *Borrelia burgdorferi* infection. The precise pathways through which *B.burgdorferi* recognition leads to inflammasome activation and processing of IL-1β in Lyme arthritis has not been elucidated. In the present study, we investigated the contribution of several pattern recognition receptors and inflammasome components in a novel murine model of Lyme arthritis.

Lyme arthritis was elicited by live *B.burgdorferi*, injected intraarticularly in knee joints of mice. To identify the relevant pathway components, the model was applied to wild-type, NLRP3-/-, ASC-/-, caspase-1-/-, NOD1-/-, NOD2-/-, and RICK-/- mice. As a control, TLR2-/-, Myd88-/- and IL-1R-/- mice were used. Peritoneal macrophages and bone marrow-derived macrophages were used for in vitro cytokine production and inflammasome activation studies. Joint inflammation was analyzed in synovial specimens and whole knee joints. Mann-Whitney U tests were used to detect statistical differences.

We demonstrate that ASC/caspase-1-driven IL-1β is crucial for induction of *B.burgdorferi*-induced murine Lyme arthritis. In addition, we show that *B.burgdorferi*-induced murine Lyme arthritis is less dependent on NOD1/NOD2/RICK pathways while the TLR2-MyD88 pathway is crucial.

Murine Lyme arthritis is strongly dependent on IL-1 production, and *B. burgdorferi* induces inflammasome-mediated caspase-1 activation. Next to that, murine Lyme arthritis is ASC and caspase-1-dependent, but NLRP3, NOD1, NOD2, and RICK independent. Also, caspase-1 activation by *B. burgdorferi* is dependent on TLR2 and MyD88. Based on present results indicating that IL-1 is one of the major mediators in Lyme arthritis, there is a rationale to propose that neutralizing IL-1 activity may also have beneficial effects in chronic Lyme arthritis.

Introduction

Lyme disease is a complex infectious disease, caused by spirochetes of the *Borrelia burgdorferi* sensu lato family. The initial host response towards *Borrelia* is mediated by the innate immune system through recognition by pattern recognition receptors (PRRs) [1-3].

Toll-like receptor 2 (TLR2) recognizes *Borrelia* species. Cells from TLR2-deficient mice show decreased cytokine production after exposure to *Borrelia* species [4], and infection with live *B.burgdorferi* in these mice results in up to 100-fold more spirochetes in their joints [5]. Cells of humans bearing a single nucleotide polymorphism (SNP) in their TLR2 gene show reduced cytokine production when exposed to *Borrelia* derived antigens [6]. Furthermore, we and others have found that TLR1/2, but not TLR2/6, heterodimers are essential for *B.burgdorferi*-dependent cytokine production [7, 8]. The crucial role for a TLR-mediated pathway was further underlined by studies using MyD88 gene deficient mice [9, 10]. When Myd88-deficient mice were injected with live *B.burgdorferi*, highly elevated spirochetal burden was found in several organs of the mice, indicating the pivotal role of Myd88 in innate host defense against *Borrelia* [11].

B.burgdorferi is recognized by the intracellular receptor nucleotide-binding oligomerization domain-containing protein 2 (NOD2), a member of the NLR family. Recently, it was demonstrated that NOD2 is needed for optimal cytokine production after *B.burgdorferi* stimulation in mice, but does not affect the spirochetal burden [12]. Cells from humans bearing the NOD2 frameshift mutation produced less IL-1β when exposed to *B.burgdorferi*, indicating that this PRR is also important in Lyme disease [3].

The pro-inflammatory cytokine interleukin (IL)-1β is known to play a major role in the pathogenesis of Lyme arthritis [13-15]. Synthesis of its inactive precursor pro-IL-1β is initiated by signals induced through PRRs [16], processing of pro-IL-1β to yield the active cytokine requires cleavage by caspase-1 [17]. In turn, caspase-1 activation needs assembly of a protein platform known as the inflammasome, of which the NLR-family member NLRP3 is the most studied [18].

In the present study, we explored the signaling pathways involved in recognition of *B.burgdorferi* by immune cells and their effect on the induction of cytokines. We investigated the two major recognition pathways for *B.burgdorferi*, TLR2-MyD88 and NOD2-RICK in the induction of Lyme arthritis. In addition, the role of components of the NLRP3 inflammasome for *Borrelia*-induced Lyme arthritis was explored.

Methods

Borrelia burgdorferi cultures. *B.burgdorferi* ATCC strain 35210, was cultured at 33°C in Barbour-Stoenner-Kelley (BSK)-H medium (Sigma-Aldrich) supplemented with 6% rabbit serum. Spirochetes were grown as described by Oosting et al [19].

Animals. IL-1R knockout mice were from Jackson Laboratories (B6.129S7-Il1r1^{tm1Imx}/J). Female wild-type (C57Bl/6J) and knock-out mice between 8-10 weeks of age were used. The mice were fed sterilized laboratory chow (Hope Farms, Woerden, The Netherlands) and water ad libitum. The protocol was approved by the Ethics Committee on Animal Experiments of the Radboud University Nijmegen Medical Centre (RU-DEC-2011-013). MyD88-/-, TLR2-/-, NOD1-/-, NOD2-/-, RICK-/-, ASC-/-, NLRP3-/-, and caspase-1-/- mice were bred and maintained in the St. Jude Children's Research Hospital, Memphis, TN, USA, as previously described [4, 20-25]. Female wild-type (C57Bl/6J) and knock-out mice between 8-10 weeks of age were used. Animal studies were conducted under protocols approved by St. Jude Children's Research Hospital Committee on Use and Care of Animals.

In vitro cytokine production. Bone marrow was isolated according to Oosting et al [19]. For stimulation of BMDMs and cytokine measurements, see additional file 3, supplementary text.

Western blot. Western Blot analysis was performed according to the procedure described by Oosting et al. [19].

Induction of *Borrelia*-induced joint inflammation and histology. Joint inflammation was induced by intra-articular injection (i.a.) of 1x10^7 live *B.burgdorferi* in 10µL of PBS into the right knee joint of naïve or knock-out mice. 4 hours after i.a. injection, mice were sacrificed and synovial specimens were isolated. After 24h, knee joints were removed for histology. Before removal of the joints, macroscopic score of the thickness of the joints (without skin) was performed ranging from no swelling (score is 0) or very severely swollen joints (score is referred as 3). Whole knee joints were removed and fixed in 4% formaldehyde for 7 days before decalcification in 5% formic acid and processing for paraffin embedding. Histology of 7 µM knee sections was performed as described before [19].

RNA isolation and real-time quantitative PCR. RNA from mouse cells was isolated using TRIzol reagent (Invitrogen) following the manufacturer's instructions. Isolated RNA was reversed transcribed into complementary DNA using iScript cDNA synthesis kit (Bio-Rad Laboratories, Veenendaal, the Netherlands). See additional file 3, supplementary text for quantitative real-time PCR.

Isolation of patella biopsies and patella washout assays. After resection of the patella with surrounding tissue, biopsies for mRNA expression assays were isolated using 3-mm disposable biopsy punches (Miltex, Integra, Germany) and immediately after isolation frozen in liquid nitrogen. Samples were stored at -80°C until RNA was extracted according to the method described above. Before RNA isolation, biopsies were lysed using the MagNALyser (Roche Applied Science, Germany). Patella washout assays were performed as described before [19].

Statistical Analysis. The data are expressed as mean ± SEM. Differences between experimental groups were tested using the two-sided Mann-Whitney U test performed on

GraphPad Prism 4.0 software (GraphPad). P values of ≤0.05 were considered significant.

Results

Induction of murine Lyme arthritis by intraarticular injection of live *B.burgdorferi.* Infection of C3H/HeN mice is a standard model for Lyme arthritis. C3H/HeN mice are highly susceptible to develop severe Lyme arthritis upon intradermal injection with *Borrelia*. C57Bl/6 mice are known to develop only mild symptoms caused by *Borrelia* species [26]. To induce murine Lyme arthritis in C57Bl/6 mice, we have used several application routes, ranging from intraperitoneal, intravenous, and intradermal in the lower back. However, none of these injection routes resulted in the development of detectable arthritis in wild-type C57/Bl6 mice (data not shown). In addition, we performed studies with dose-ranges up to $1x10^7$ spirochetes per injection. No signs of *Borrelia*-induced joint inflammation were seen in the C57/Bl6 mice (data not shown).

It has been demonstrated in patients with Lyme arthritis that *Borrelia* spirochetes were detected in synovial-fluid or -tissue using either PCR or culture [27, 28]. Therefore, we injected live *Borrelia* directly into knee joints of C57Bl/6 mice to mimic the clinical practice of patients with active Lyme arthritis. We were able to induce joint inflammation resulting in joint swelling and cell influx into the joint cavity up to day 7 after intra articular (i.a.) of spirochetes (Figure 1A/B). Using this novel model of Lyme arthritis we could address the goal of the current study, to investigate the upstream mediators of *Borrelia* burgdorferi-induced activation of the inflammasome and the contribution of individual components of the inflammasome in Lyme arthritis.

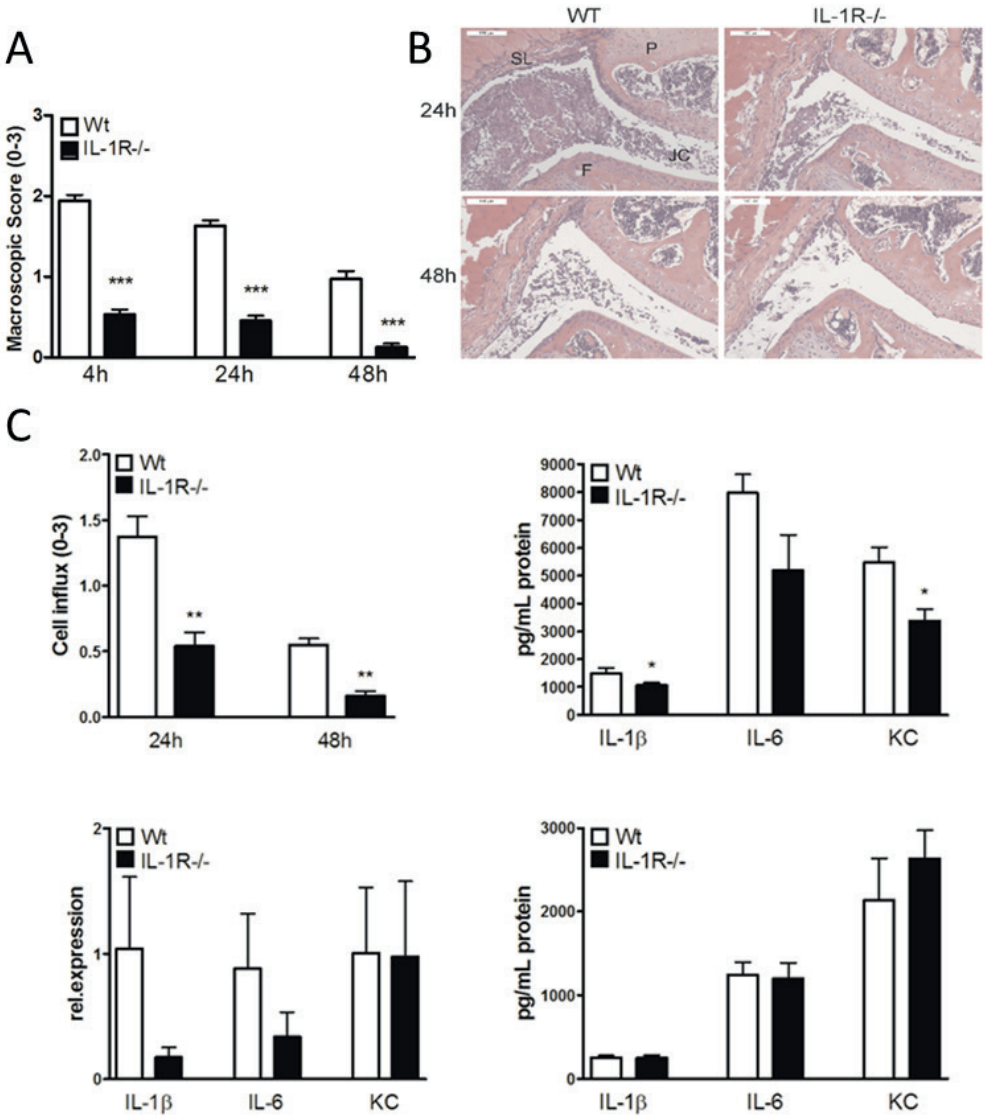
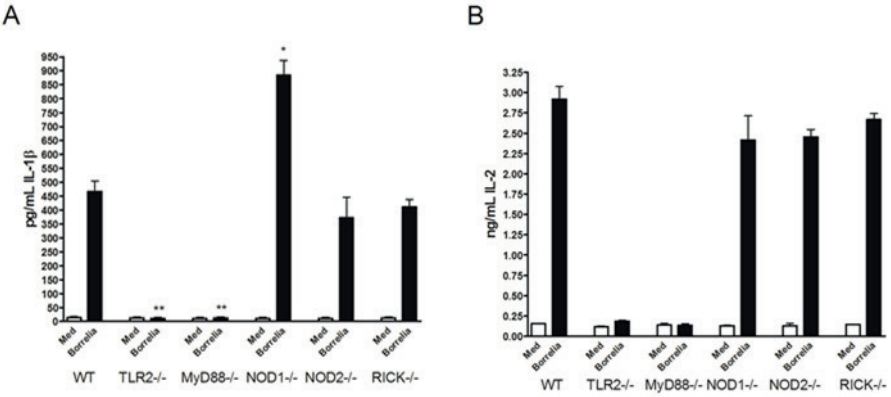


Figure 1. Murine Lyme arthritis is IL-1 dependent. (A) Macroscopic score of the knees in either wild-type (white bars), or IL-1Receptor-/- mice (black bars). After 4, 24, and 48 hours of intra-articular injection of 1x10^7 live *B.burgdorferi*, at least 10 knees per group. Data are mean±SEM from 8 animals in each group; ***p<0.0001; Mann–Whitney U test, two-tailed. (B) Murine Lyme arthritis in WT, or IL-1R-/- mice. Histology (H&E staining) 24, and 48 hours after i.a. injection of *B.burgdorferi* in knee joints. 200x magnification; P, patella; F, femur; JC, joint cavity; SL, synovial lining. Scale bar represents 100 μM. (C) Upper left: Scored cell influx after 24 and 48 hours of i.a. injection with *B.burgdorferi*. Upper right and lower left: 4 hours after i.a. injection of 1x10^7 live *B.burgdorferi* in 10 μL of PBS, patellae were cultured for 1h and IL-1β, IL-6 and KC protein levels and mRNA expression levels were measured using Luminex and qPCR, respectively. Lower right: After 24 hours of infection, 1x10^5 peritoneal macrophages were stimulated for 24 hours with live *B.burgdorferi*. White bars represent cytokine induction by wild-type mice, black bars are IL-1R gene deficient mice, at least 5 animals/group. *p<0.05, **p<0.01; Mann-Whitney U-test, two-sided.

Murine Lyme arthritis is strongly dependent on IL-1 production. IL-1 was shown already to play an important role in the pathogenesis of Lyme disease, but the role in the development of murine Lyme arthritis was never been described so far [15]. To corroborate the role of IL-1 in the induction of our novel murine model of Lyme arthritis, IL-1R-deficient mice were injected intraarticularly (i.a.) with live *B.burgdorferi.* Compared to wild-type mice, IL-1R-/- mice exhibited significantly reduced joint swelling at early (4h) and late (48h) time points (Figure 1A). This was reflected by histology: IL-1R gene deficient mice displayed a considerable reduction in the numbers of inflammatory cells in the joint cavity when compared to wild-type mice (Figure 1B). At 24h and 48h after induction of Lyme arthritis, the cell influx was decreased in IL-1R-/- mice (Figure 1C, upper left). In these mice, significantly reduced protein concentrations of IL-1β and the chemokine KC were found in patella washouts (Figure 1C, upper right). Synovial tissue explants of IL-1R-/- mice showed less IL-1β, and IL-6 mRNA expression, 4 hours after induction of Lyme arthritis (Figure 1C, lower left). KC mRNA expression was found to be similar between wild-type and IL-1R-/- mice (Figure 1C, lower left). No differences between wild-type and IL-1R knockout mice were observed in IL-1β, IL-6, and KC production of peritoneal macrophages stimulated with *B.burgdorferi* (Figure 1C, lower right).

***Borrelia*-induced IL-1β production is NOD1/2 and RICK independent.** In humans, *B.burgdorferi*-induced IL-1β was partly NOD2-dependent, but the role of this PRR in the development of arthritis was never been demonstrated. The roles of murine NOD1, NOD2, and RICK were explored using mouse cells. BMDMs isolated from wild-type, NOD1, NOD2, or RICK deficient mice were stimulated with either medium or *B.burgdorferi*. A clear induction of mRNA coding for IL-1β was seen in wild-type BMDMs upon stimulation with *B.burgdorferi* (Additional file 1, Figure S1). Surprisingly, NOD1, NOD2, and RICK appeared not to be important for the induction of IL-1β after *B.burgdorferi* recognition, whereas – as expected – TLR2 and MyD88 were (Figure 2A). In the absence of NOD1, IL-1β mRNA as well as IL-1β protein levels were higher than in cells of wild-type mice, indicating an inhibitory role of NOD1 in *B.burgdorferi*-induced IL-1β production (Additional file 1 and 2, Figure S1 and S2A). The IL-1 induced by BMDMs was bioactive in the IL-2 induction assay (Figure 2B). NOD1-, NOD2- and RICK-deficient cells induced IL-6 and TNF-α production after exposure to *B.burgdorferi* (Additional file 1, Figure S1).

TLR2 and MyD88 mediated pathways are crucial for the *Borrelia*-induced IL-1β production. BMDMs isolated from wild-type, TLR2-/-, and MyD88-/- mice were exposed to either medium or *B.burgdorferi*. TLR2 and MyD88 are not only crucial for the induction of IL-1β-mRNA, but also important for the *B.burgdorferi*-induced IL-1β protein secretion in the supernatant (Additional file 1, Figure S1, and Additional file 2, Figure S2A).
The IL-1β induced by BMDMs from wild-type mice was bioactive in the IL-2 assay, whereas no bioactive IL-1 production was seen by BMDMs of TLR2-/- and MyD88-/- mice (Figure 2B). In line with previous results, TLR2 and MyD88 are also critical for *B.burgdorferi*-induced IL-6 and TNF-α production on both protein as mRNA level (Additional file 1, Figure S1B/C).



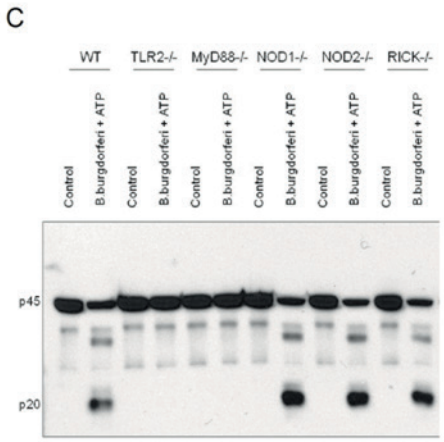


Figure 2. TLR2 and MyD88 drive *Borrelia*-induced IL-1β production. (A) 0.5x10^6 BMDMs from wild-type (WT), TLR2-/-, MyD88-/-, NOD1-/-, NOD2-/-, and RICK-/- mice were stimulated for 24 hours with either medium (Med), or 5x10^6 spirochetes per mL live *B.burgdorferi* (black bars. IL-1β protein levels in supernatant expressed in picograms per mL after BMDM exposure for 24 hours to B.burgdorferi. BMDMs are isolated from at least 5 animals per group, protein measurements were performed in duplicates. (B) IL-2 protein levels in nanograms per mL after BMDM stimulation with medium (Med), or 5x10^6 *B.burgdorferi* per mL for 24 hours, using cells from different knockout mice. (C) 3x10^6 Bone marrow derived macrophages from 5 WT C57Bl/6, TLR2-/-, MyD88-/-, NOD1-/-, NOD2-/-, or RICK-/- mice were incubated for 24 hours with or without 1x10^6 *B.burgdorferi* with ATP (3mM) for 30 minutes. Cleaved caspase-1 was detected by Western Blotting using antibodies to detect the inactive caspase-1 (p45) or active caspase-1 (p20). The control conditions were already published in an earlier article by our group [19]. *Borrelia* and ATP by itself are unable to induce caspase-1 activation, the combination of the two are crucial for inducing cleavage.

Caspase-1 activation by *B.burgdorferi* is dependent on TLR2 and MyD88. Inactive pro-caspase-1 needs to be cleaved to yield active caspase-1 before it can process pro-IL-1β. To identify the signaling cascades involved in *B.burgdorferi*-induced caspase-1 activation, BMDMs of TLR2-, MyD88-, NOD1-, NOD2-, or RICK-deficient mice were exposed to either medium (control) or *B.burgdorferi* plus ATP. Thereafter, Western Blot analysis was performed using a specific antibody detecting the active subunit of caspase-1. Wild-type cells stimulated with *B.burgdorferi* and ATP expressed the cleaved caspase-1 (Figure 2C). Surprisingly, activation of caspase-1 in BMDMs by *B.burgdorferi* is entirely dependent on TLR2 and Myd88 mediated pathways, whereas NOD1, NOD2, and RICK signaling pathways are not required for caspase-1 activation (Figure 2C).

***B.burgdorferi* induces murine Lyme arthritis through TLR2 and MyD88.** As described before, both TLR2 and MyD88 play a critical role in *B.burgdorferi*-induced caspase-1 activation and subsequent IL-1β production in vitro. To assess the roles of these molecules in vivo, we induced Lyme arthritis by injecting live *B.burgdorferi* spirochetes into knee joints of wild-type, TLR2-, or MyD88-deficient mice. In addition, we investigated NOD1, NOD2, and RICK knockout mice. Lyme arthritis, detected as joint swelling of the injected knee, could be clearly seen in wild-type mice. Both TLR2- and MyD88-deficient mice displayed significantly less joint swelling 4 hours after induction of Lyme arthritis (Figure 3A). The lack of joint swelling was still noticeable after 24 hours (Figure 3A). Interestingly, NOD1 knockout mice displayed severe swelling, similar to that seen in wild-type mice. The expression of Lyme arthritis in NOD2 and RICK knockout mice was significantly lower than in wild-type animals, both at early (4h) and late (24h) phases (Figure 3A). Next to joint swelling, the cell influx into the joint cavity at 24h was assessed. In wild-type mice, the synovial lining was thickened and more cells, mainly neutrophils infiltrated into the joint cavity (Figure 3B and 3C). In both TLR2-, and MyD88-gene deficient mice the synovial lining was less inflamed and significant reduced cell influx could be observed (Figure 3B and 3C).

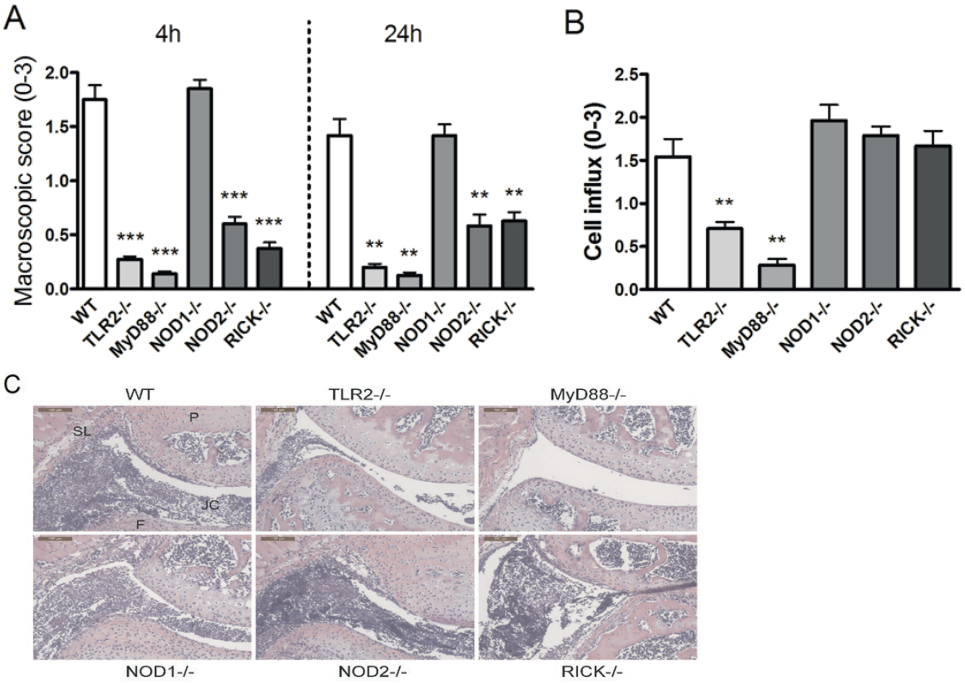


Figure 3. *B.burgdorferi*-induced murine Lyme arthritis is dependent on TLR2 and MyD88. (A) Macroscopic score of the knees in WT, TLR2ko, MyD88ko, NOD1ko, NOD2ko, and RICKko mice. After 4 hours and 24 hours of intra-articular injection of 1x10^7 live *B.burgdorferi,* at least 10 knees per group. Data are mean±SEM from 10 knees in each group; **p<0.01; ***p<0.0001, Mann–Whitney U test, two-tailed. (B) Scored cell influx 1 day after i.a. injection of *B.burgdorferi*. Data are mean±SEM from 10 knees in each group; **p<0.01; Mann–Whitney U test, two-tailed. (C) Murine Lyme arthritis in WT, TLR2ko, MyD88ko, NOD1ko, NOD2ko, and RICKko mice. Histology (H&E staining) 1 day after i.a. injection of *B.burgdorferi* in knee joints. 200x magnification; P, patella; F, femur; JC, joint cavity; SL, synovial lining. Scale bar represents 100 μM.

Inflammasome-mediated activation of caspase-1 by *B.burgdorferi*. The roles of inflammasome components ASC, and NLRP3 in the *B.burgdorferi*-induced caspase-1 activation were assessed. Caspase-1 activation could be clearly detected when BMDMs of wild-type mice were exposed to *B.burgdorferi*, but was completely absent in cells from mice deficient in NLRP3, ASC, or caspase-1 (Figure 4A). Subsequently, NLRP3, ASC, and caspase-1 are crucial for total IL-1β protein production after *B.burgdorferi* stimulation (Figure 4B). Interestingly, a significantly decreased concentration of bioactive IL-1 could be observed in absence of ASC, NLRP3, or caspase-1, as compared to wild-type mice (Figure 4C). Transcription of IL-1β mRNA was not influenced by deficiency of the inflammasome components after *B.burgdorferi* stimulation of BMDMs (Additional file 2, Figure S2A). No differences in mRNA and protein levels of both IL-6 and TNF-α could be detected between wild-type, ASC-, NLRP3- or caspase-1-deficient BMDM after stimulation with *B.burgdorferi* (Additional file 2, Figure S2B/C).

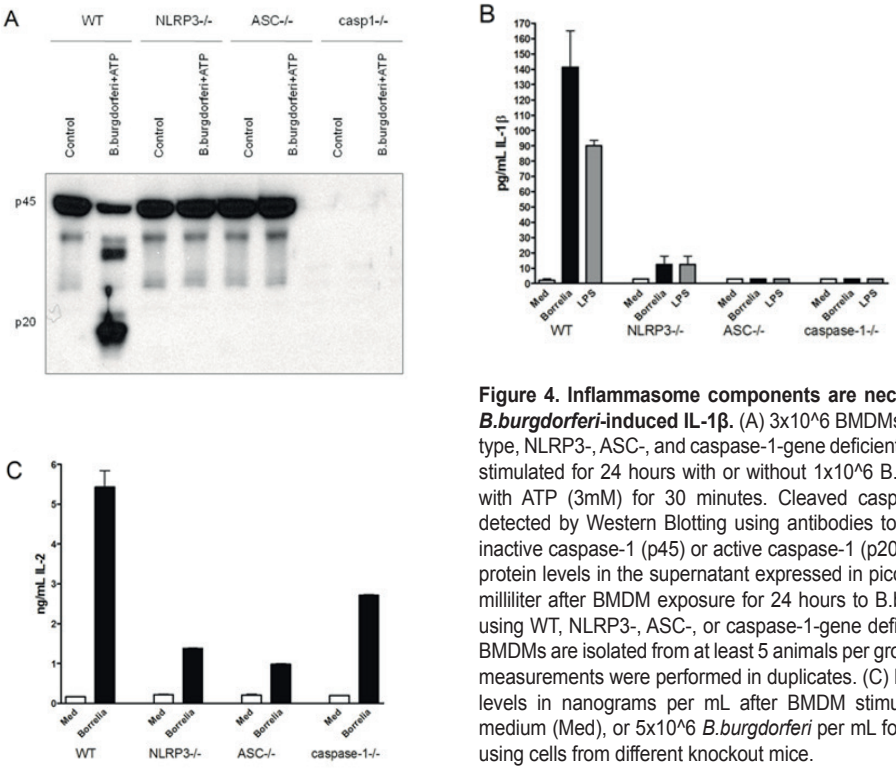


Figure 4. Inflammasome components are necessary for *B.burgdorferi*-induced IL-1β. (A) 3x10^6 BMDMs from wild-type, NLRP3-, ASC-, and caspase-1-gene deficient mice were stimulated for 24 hours with or without 1x10^6 B.burgdorferi with ATP (3mM) for 30 minutes. Cleaved caspase-1 was detected by Western Blotting using antibodies to detect the inactive caspase-1 (p45) or active caspase-1 (p20). (B) IL-1β protein levels in the supernatant expressed in picograms per milliliter after BMDM exposure for 24 hours to B.burgdorferi, using WT, NLRP3-, ASC-, or caspase-1-gene deficient mice. BMDMs are isolated from at least 5 animals per group, protein measurements were performed in duplicates. (C) IL-2 protein levels in nanograms per mL after BMDM stimulation with medium (Med), or 5x10^6 *B.burgdorferi* per mL for 24 hours, using cells from different knockout mice.

Murine Lyme arthritis is ASC and caspase-1-dependent, but NLRP3 independent. To assess the role of the inflammasome components in vivo, wild-type (WT) mice, NLRP3-/-, ASC-/-, and caspase-1-/- mice were injected with live *B.burgdorferi* and joint swelling was assessed after 4 and 24 hours (Figure 5A). ASC and caspase-1 knockout mice showed significantly reduced joint swelling after local *B.burgdorferi* injection, whereas NLRP3-gene deficient mice displayed joint inflammation similar to wild-type animals (Figure 5A).

ASC and caspase-1 knockout mice had also less thickened synovial linings than WT animals, and less cell influx into the joint cavity could be observed in these mice (Figure 5B/C). Of interest, NLRP3 is not critical in the induction of murine Lyme arthritis.

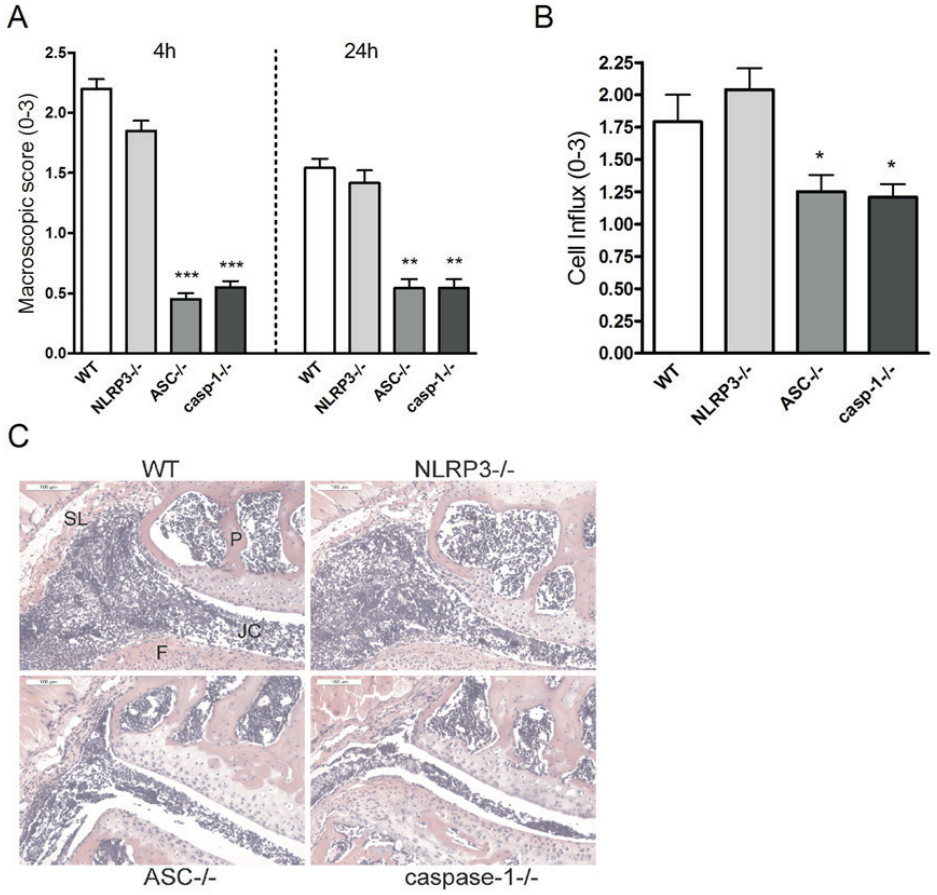
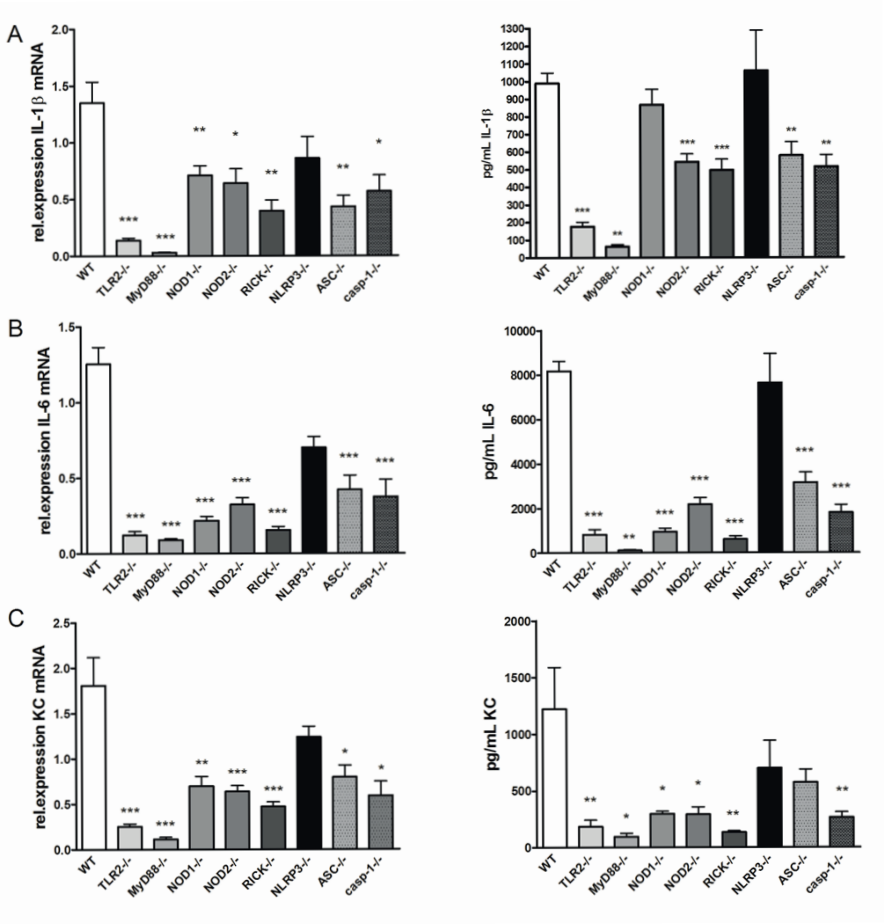


Figure 5. ASC and caspase-1- dependent role in murine Lyme arthritis. (A) Macroscopic score of the knees in WT, NLRP3, ASC, and caspase-1 knockout mice. After 4 hours and 24 hours of intra-articular injection of 1x10^7 live *B.burgdorferi*, at least 10 knees per group. Data are mean±SEM from 10 knees in each group; **p<0.01; ***p<0.0001; Mann–Whitney U test, two-tailed. (B) Scored cell influx 1 day after i.a. injection of *B.burgdorferi*. Data are mean±SEM from 10 knees in each group; *p<0.05; Mann–Whitney U test, two-tailed. (C) Murine Lyme arthritis in WT, NLRP3, ASC, and caspase-1 knockout mice. Histology (H&E staining) 1 day after i.a. injection of *B.burgdorferi* in knee joints. 200x magnification; P, patella; F, femur; JC, joint cavity; SL, synovial lining. Scale bar represents 100 µM.

NLRP3-independent local cytokine production after *B.burgdorferi* injection. The strong reduction of arthritis was in line with the findings that the local cytokine production was ablated in both TLR2-, and MyD88-deficient mice (Figure 6). Synovial tissue explants revealed that both the mRNA expression as the protein production of IL-1β, IL-6, and KC was almost absent in TLR2 and MyD88 knockout mice (Figure 6A/C). A remarkable finding was the fact that NOD1, NOD2, and RICK signaling does not seem to be involved

in the cell influx as similar numbers of inflammatory cells were found in the joint cavity as in wild-type mice (Figure 3B). This is in sharp contrast to the strongly reduced synovial production of IL-6, and KC in NOD1-/-, NOD2-/-, and RICK-/- mice, although NOD1 deficient mice produced similar amounts of IL-1β as wild-type mice and NOD2-/- and RICK-/- mice showed only 50% reduction in IL-1β (Figure 6). The inhibitory effect of NOD1 seen in figure 3 for *Borrelia*-induced IL-1β production could not be observed using BMDM stimulation.

Next, we examined the local cytokine production in patellae after injection of *B.burgdorferi*. Levels of IL-1β, IL-6, and KC mRNA expression in synovial tissue of ASC and caspase-1 deficient mice was found to be significantly lower, with exception of NLRP3 deficient mice as compared to wild-type mice (Figure 6A). Similar results were observed for synovial IL-6 and KC mRNA expression (Figure 6B/C). Cytokine measurements in patella washouts showed that IL-1β production by the inflamed synovium was dependent on ASC and caspase-1, but not on NLRP3 (Figure 6A). This was also true for induction of IL-6, and KC (Figure 6B/C). Both ASC and caspase-1 are pivotal components for the induction of the latter cytokines.



← page 32

Figure 6. TLR2 and MyD88 dependent and NLRP3 independent local cytokine production in synovium. (A) IL-1β mRNA expression and protein levels, IL-6 mRNA expression and protein (B), or KC mRNA expression and protein (C), in biopsies isolated from synovial tissues from either wild-type, TLR2-, MyD88-, NOD1-, NOD2-, RICK-, NLRP3-, ASC-, and caspase-1-gene deficient mice. 4 hours after intra-articular injection with 1x10^7 live *B.burgdorferi*. At least 5 animals per group, bars represent mean±SEM; *p<0.05; **p<0.01; ***p<0.0001, Mann-Whitney U-test, two-sided. (A-C Right panels) Four hours after intra-articular (i.a.) injection of 1x10^7 live *B.burgdorferi* in 10 mL of PBS, patellae from 5 WT C57BL/6 mice or knockout mice were cultured for 1h and IL-1β (A), IL-6 (B), and KC (C) levels were measured using Luminex. Data are represented as mean±SEM; 5 animals in each group; **p<0.01, ***p<0.001; Mann–Whitney U test, two-tailed.Scale bar represents 100 μM.

Discussion

In the present study, we describe for the first time the role of TLR2 and MyD88 in vivo in a murine model of Lyme arthritis elicited by local injection of *B.burgdorferi*. TLR2 and MyD88 are crucial for *B.burgdorferi*-dependent caspase-1 activation and subsequent IL-1β production in vitro by bone marrow-derived macrophages. Mice deficient in TLR2 or MyD88 display less joint swelling after intra-articular injection of *B.burgdorferi* and fewer cells are attracted towards the joint cavity. In addition to TLR2 and MyD88, the inflammasome components ASC and caspase-1 are essential for IL-1β production by *B.burgdorferi*-stimulated BMDMs in vitro, and also are pivotal in the development of murine Lyme arthritis. The NOD1/NOD2/RICK pathway is also involved in synovial cell activation by *B.burgdorferi*, but is not essential for the production of IL-1β and for the influx of inflammatory cells into the joint cavity.

It is known that genetic background of mice influences the susceptibility for several experimental disease models [29, 30]. This holds also true for the induction and maintenance of experimental murine Lyme Disease. C3H/HeN mice are highly susceptible to develop severe arthritis upon intradermal injection with *Borrelia*. However, these C3H/HeN mice also display a defect in IL-12 production and therefore lack IL-12/IL-18 induced IFN-γ production upon stimulation with pathogens [31]. This is in contrast to cells from C57Bl/6 mice which produce IFN-γ after *Borrelia* exposure [19]. This might explain the susceptibility of the C3H/HeN mice to the development of Lyme arthritis. Lacking IFN-γ production results in less killing of invading *Borrelia* bacteria, resulting in dissemination through the body. In our study, we used C57Bl/6 mice, which do not display any defects in immune responses and therefore will reflect the disease development in humans more appropriate.

It has been suggested earlier [15], that IL-1 is a key player in the pathogenesis of Lyme arthritis. Patients with Lyme arthritis who were found to have elevated synovial concentrations of IL-1Receptor antagonist (IL-1Ra) in combination with low concentrations of IL-1β showed a rapid resolution of Lyme arthritis. These data are in line with the results of the present study in murine Lyme arthritis. Mice deficient for the IL-1 receptor showed

significantly attenuated *B.burgdorferi*-induced arthritis. In addition, decreased levels of IL-1β were noted in mice without functional IL-1R. Both mRNA expression and protein concentrations of IL-1β were lower in synovial cells lacking IL-1R, when compared to wild-type mice. These findings point towards an amplification loop in the local production of IL-1β via its receptor during the onset of Lyme arthritis.

Before active IL-1β can be secreted, the inflammasome needs to be assembled intracellularly by heteromultimerization [17]. NLRP3 is seen as one of the major NLRs that activate the inflammasome, followed by the cleavage of pro-caspase-1. In our hands, using caspase-1 deficient mice, caspase-1 seems to be the major enzyme to cleave pro-IL-1β. However, at later time points after *Borrelia* exposure, caspase-1 might play a different role. Liu et al., [32] showed that caspase-1 knockout mice display higher arthritis scores at day 14 post infection. On day 45, the arthritis scores in these mice are lower as compared to wild-type mice. However, the inoculation method between this study and our data differs and might cause a different amount of spirochetes in the joint at early time points. Next to this, recently it was demonstrated that the caspase-1 knockout mice used in these studies were in fact double knock-out, they also lack functional caspase-11 [33]. Recently, it was demonstrated that mice deficient for only caspase-1 were more susceptible for intracellular infections [34]. The caspase-1/11 deficient mice were less susceptible, indicating that caspase-11 may dampen the effect of caspase-1 in our findings. Taken this together, caspase-1 plays an important role in the induction of an inflammatory response against *Borrelia* spirochetes in the joint, but is less important in controlling spirochete burden at later time points.

Here we show that in vitro activation of caspase-1 in BMDMs after *B.burgdorferi* exposure, as well as production of IL-1β protein and bioactive IL-1β, is indeed NLRP3-dependent (Fig.4). However, intraarticular injection of *B.burgdorferi* did not differ between NLRP3 knockout and wild-type mice in terms of joint inflammation and cytokine production. These results support recent reports showing that NLRP3 is not involved in other murine models of arthritis, such as antigen-induced arthritis, collagen-induced arthritis, or gouty arthritis [35-37]. We cannot fully explain the difference in NLRP3 dependency between the in vitro and in vivo induction of IL-1β by *B.burgdorferi*. Explanations may be sought in in vivo activation of additional inflammasomes, or triggering of ASC-caspase-1 independently of NLRs [38-40], but this remains to be demonstrated.

Interestingly, ASC was found to be pivotal in the induction of Lyme arthritis. It is known that ASC is not just an adaptor protein within the inflammasome, but also has a role in antigen presentation and lymphocyte migration. It addition, it has been demonstrated that ASC controls mRNA stability and expression of Dock2, which is involved in Rac activation in immune cells [41]. Finally, ASC has been associated with NFκB signaling [42], which is the major pathway in the production of several cytokines.

In contrast to TLR2-MyD88, neither NOD1 nor NOD2 were involved in the *B.burgdorferi*-triggered caspase-1 activation and bioactive IL-1β production in BMDMs (Fig.2B/C).

However, in vivo we note a NOD1/NOD2/RICK-dependent cytokine production in synovial explants from mice with Lyme arthritis (Fig.6). The latter findings are in line with a recent report [3] in humans lacking functional NOD2, which show that *Borrelia*-induced IL-1β production is partly NOD2 dependent. Apparently the recognition of *B.burgdorferi* by murine or human immune cells through PRRs differs. Although earlier results demonstrate a specific role for RICK and NOD2 in the recognition and induction of *Borrelia*-dependent IL-1β production by human cells, we were unable to detect the important role for NOD2 in vivo for cell influx in murine Lyme arthritis [3]. However, the role of these PRRs in the in vitro cytokine induction using murine cells could be confirmed in the present study.

An inhibitory role for NOD1 could be demonstrated in this manuscript, but was not found when studying human cytokine responses upon *Borrelia* stimulation. These discrepant observations might certainly be explained by differences between host species, it is described that humans express different PRR and explore different methods to induce a proper immune response against pathogens. Murine cells produce pro-inflammatory cytokines after *B.burgdorferi* exposure, such as IL-1β, KC, and TNF-α. Whether this difference is caused by hyperactive TLR2 signaling or lack of NOD2 mediated suppression is not elucidated yet.

The role of MyD88 in experimental Lyme disease, but also in the host response against *B.burgdorferi* was studied previously [43-45]. MyD88 deficient mice displayed severely higher amounts of spirochetes in several tissues, including the joints [11, 43]. It was shown that MyD88 knockout mice infected with live *B.burgdorferi* displayed more severe arthritis and cell influx as compared to infected wild-type mice [43]. Therefore, it was concluded in this study that Lyme arthritis occurs without the presence of the MyD88 molecule. However, these findings can be explained by the fact that MyD88 deficient mice suffer from a higher spirochetal burden than wild-type mice. The excessive spirochetal load in the organs causes hyperinflammation that is MyD88-independent.

In the present study we detected an important role for MyD88 in the development of early murine Lyme arthritis. Mice deficient in this adaptor molecule were not able to develop arthritis after intra-articular injection of *B.burgdorferi*. TLR2 gene deficient mice were also unable to induce Lyme arthritis at early time points after injection of spirochetes. These data suggest that TLR2-MyD88 signaling is very important during the onset of Lyme arthritis.

The treatment of patients suffering from Lyme arthritis is a challenge for clinicians, as treatment is often ineffective. Patients suffering from gout, rheumatoid arthritis, or other inflammatory joint diseases benefit from treatment with the IL-1receptor antagonist (Anakinra) [46, 47]. Based on present results indicating that IL-1 is one of the major mediators in Lyme arthritis, there is a rationale to propose that neutralizing IL-1 activity may also have beneficial effects in chronic Lyme arthritis. Apart from IL-1Ra, anti-IL-1β antibodies like Canakinumab might be useful for treatment of Lyme disease since these antibodies express a long half-life in humans. Thus, understanding the precise

pathogenesis of Lyme disease may reveal novel therapeutic modalities in the near future.

Conclusions

In the present study, we have demonstrated that murine Lyme arthritis is strongly dependent on IL-1 production. Next to that, murine Lyme arthritis is ASC and caspase-1-dependent, but NLRP3, NOD1, NOD2, and RICK independent. Caspase-1 activation by *B. burgdorferi* is dependent on TLR2 and MyD88.

In light of these findings, we propose a critical role for TLR2-MyD88-NLR-ASC-caspase-1 cascade mediated IL-1β production in the pathogenesis of Lyme arthritis. The treatment of patients suffering from Lyme arthritis is still challenging for clinicians, and treatment for patients with chronic Lyme disease is often ineffective. Based on the present results indicating that IL-1 is one of the major mediators in Lyme arthritis, it is rationale to propose that neutralizing IL-1 activity may also have beneficial effects in chronic Lyme arthritis.

Acknowledgements

We thank P. Vandenabeele (Ghent University, Ghent, Belgium) for the generous supply of Rabbit anti-mouse caspase-1 antibody. Monique M. Helsen (Department of Rheumatology) is acknowledged for excellent histology. We also would like to thank Carla Bartels (Department of Medical Microbiology, Radboud University Nijmegen Medical Centre), for culturing and counting Borrelia spirochetes.

References

1. Berende A, Oosting M, Kullberg BJ, Netea MG, Joosten LA: **Activation of innate host defense mechanisms by Borrelia**. *EurCytokine Netw* 2010, **21**:7-18.

2. Cervantes JL, Dunham-Ems SM, La Vake CJ, Petzke MM, Sahay B, Sellati TJ, Radolf JD, Salazar JC: **Phagosomal signaling by Borrelia burgdorferi in human monocytes involves Toll-like receptor (TLR) 2 and TLR8 cooperativity and TLR8-mediated induction of IFN-{beta}**. *ProcNatlAcadSciUSA* 2011, **108**:3683-3688.

3. Oosting M, Berende A, Sturm P, Ter Hofstede HJ, de Jong DJ, Kanneganti TD, Van der Meer JW, Kullberg BJ, Netea MG, Joosten LA: **Recognition of Borrelia burgdorferi by NOD2 is central for the induction of an inflammatory reaction**. *JInfectDis* 2010, **201**:1849-1858.

4. Wooten RM, Ma Y, Yoder RA, Brown JP, Weis JH, Zachary JF, Kirschning CJ, Weis JJ: **Toll-like receptor 2 is required for innate, but not acquired, host defense to Borrelia burgdorferi**. *JImmunol* 2002, **168**:348-355.

5. Wang G, Ma Y, Buyuk A, McClain S, Weis JJ, Schwartz I: **Impaired host defense to infection and Toll-like receptor 2-independent killing of Borrelia burgdorferi clinical isolates in TLR2-deficient C3H/HeJ mice**. *FEMS MicrobiolLett* 2004, **231**:219-225.

6. Schroder NW, Diterich I, Zinke A, Eckert J, Draing C, von B, V, Hassler D, Priem S, Hahn K, Michelsen KS *et al*: **Heterozygous Arg753Gln polymorphism of human TLR-2 impairs immune activation by Borrelia burgdorferi and protects from late stage Lyme disease**. *JImmunol* 2005, **175**:2534-2540.

7. Ozinsky A, Underhill DM, Fontenot JD, Hajjar AM, Smith KD, Wilson CB, Schroeder L, Aderem A: **The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors**. *ProcNatlAcadSciUSA* 2000, **97**:13766-13771.

8. Oosting M, Ter HH, Sturm P, Adema GJ, Kullberg BJ, Van der Meer JW, Netea MG, Joosten LA: **TLR1/TLR2 Heterodimers Play an Important Role in the Recognition of Borrelia Spirochetes**. *PLoSOne* 2011, **6**:e25998.

9. Kawai T, Akira S: **TLR signaling**. *Cell DeathDiffer* 2006, **13**:816-825.

10. Dennis VA, Dixit S, O'Brien SM, Alvarez X, Pahar B, Philipp MT: **Live Borrelia burgdorferi spirochetes elicit inflammatory mediators from human monocytes via the Toll-like receptor signaling pathway**. *InfectImmun* 2009, **77**:1238-1245.

11. Behera AK, Hildebrand E, Bronson RT, Perides G, Uematsu S, Akira S, Hu LT: **MyD88 deficiency results in tissue-specific changes in cytokine induction and inflammation in interleukin-18-independent mice infected with Borrelia burgdorferi**. *InfectImmun* 2006, **74**:1462-1470.

12. Petnicki-Ocwieja T, Defrancesco AS, Chung E, Darcy CT, Bronson RT, Kobayashi KS, Hu LT: **Nod2 Suppresses Borrelia burgdorferi Mediated Murine Lyme Arthritis and Carditis through the Induction of Tolerance**. *PLoSOne* 2011, **6**:e17414.

13. Beck G, Habicht GS, Benach JL, Coleman JL, Lysik RM, O'Brien RF: **A role for interleukin-1 in the pathogenesis of Lyme disease**. *ZentralblBakteriolMikrobiolHygA* 1986, **263**:133-136.

14. Habicht GS, Beck G, Benach JL: **The role of interleukin-1 in the pathogenesis of Lyme disease**. *AnnNYAcadSci* 1988, **539**:80-86.

15. Miller LC, Lynch EA, Isa S, Logan JW, Dinarello CA, Steere AC: **Balance of synovial fluid IL-1 beta and IL-1 receptor antagonist and recovery from Lyme arthritis**. *Lancet* 1993, **341**:146-148.

16. Dinarello CA: **Blocking IL-1 in systemic inflammation**. *JExpMed* 2005, **201**:1355-1359.

17. Eder C: **Mechanisms of interleukin-1beta release**. *Immunobiology* 2009, **214**:543-553.

18. van d, V, Netea MG, Dinarello CA, Joosten LA: **Inflammasome activation and IL-1beta and IL-18 processing during infection**. *Trends Immunol* 2011, **32**:110-116.

19. Oosting M, van d, V, Kanneganti TD, Sturm P, Verschueren I, Berende A, Van der Meer JW, Kullberg BJ, Netea MG, Joosten LA: **Borrelia species induce inflammasome activation and IL-17 production through a caspase-1-dependent mechanism**. *EurJImmunol* 2011, **41**:172-181.

20. Adachi O, Kawai T, Takeda K, Matsumoto M, Tsutsui H, Sakagami M, Nakanishi K, Akira S: **Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function**. *Immunity* 1998, **9**:143-150.

21. Chamaillard M, Hashimoto M, Horie Y, Masumoto J, Qiu S, Saab L, Ogura Y, Kawasaki A, Fukase K, Kusumoto S *et al*: **An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid**. *NatImmunol* 2003, **4**:702-707.

22. Kanneganti TD, Ozoren N, Body-Malapel M, Amer A, Park JH, Franchi L, Whitfield J, Barchet W, Colonna M, Vandenabeele P *et al*: **Bacterial RNA and small antiviral compounds activate caspase-1 through cryopyrin/Nalp3**. *Nature* 2006, **440**:233-236.

23. Kobayashi K, Inohara N, Hernandez LD, Galan JE, Nunez G, Janeway CA, Medzhitov R, Flavell RA: **RICK/Rip2/CARDIAK mediates signalling for receptors of the innate and adaptive immune systems**. *Nature* 2002, **416**:194-199.

24. Kobayashi KS, Chamaillard M, Ogura Y, Henegariu O, Inohara N, Nunez G, Flavell RA: **Nod2-dependent regulation of innate and adaptive immunity in the intestinal tract**. *Science* 2005, **307**:731-734.

25. Shaw PJ, Lukens JR, Burns S, Chi H, McGargill MA, Kanneganti TD: **Cutting edge: critical role for PYCARD/ASC in the development of experimental autoimmune encephalomyelitis**. *JImmunol* 2010, **184**:4610-4614.

26. Ganapamo F, Dennis VA, Philipp MT: **Differential acquired immune**

responsiveness to bacterial lipoproteins in Lyme disease-resistant and -susceptible mouse strains. *EurJImmunol* 2003, **33**:1934-1940.

27. Brisson D, Baxamusa N, Schwartz I, Wormser GP: **Biodiversity of Borrelia burgdorferi strains in tissues of Lyme disease patients**. *PLoSOne* 2011, **6**:e22926.

28. Li X, McHugh GA, Damle N, Sikand VK, Glickstein L, Steere AC: **Burden and viability of Borrelia burgdorferi in skin and joints of patients with erythema migrans or lyme arthritis**. *Arthritis Rheum* 2011, **63**:2238-2247.

29. Armstrong AL, Barthold SW, Persing DH, Beck DS: **Carditis in Lyme disease susceptible and resistant strains of laboratory mice infected with Borrelia burgdorferi**. *AmJTropMedHyg* 1992, **47**:249-258.

30. Barthold SW, Beck DS, Hansen GM, Terwilliger GA, Moody KD: **Lyme borreliosis in selected strains and ages of laboratory mice**. *JInfectDis* 1990, **162**:133-138.

31. Balkhy HH, Heinzel FP: **Endotoxin fails to induce IFN-gamma in endotoxin-tolerant mice: deficiencies in both IL-12 heterodimer production and IL-12 responsiveness**. *JImmunol* 1999, **162**:3633-3638.

32. Liu N, Belperron AA, Booth CJ, Bockenstedt LK: **The caspase 1 inflammasome is not required for control of murine Lyme borreliosis**. *Infection and immunity* 2009, **77**:3320-3327.

33. Kayagaki N, Warming S, Lamkanfi M, Vande Walle L, Louie S, Dong J, Newton K, Qu Y, Liu J, Heldens S *et al*: **Non-canonical inflammasome activation targets caspase-11**. *Nature* 2011, **479**:117-121.

34. Broz P, Ruby T, Belhocine K, Bouley DM, Kayagaki N, Dixit VM, Monack DM: **Caspase-11 increases susceptibility to Salmonella infection in the absence of caspase-1**. *Nature* 2012.

35. Kolly L, Karababa M, Joosten LA, Narayan S, Salvi R, Petrilli V, Tschopp J, van den Berg WB, So AK, Busso N: **Inflammatory role of ASC in antigen-induced arthritis is independent of caspase-1, NALP-3, and IPAF**. *JImmunol* 2009, **183**:4003-4012.

36. Ippagunta SK, Brand DD, Luo J, Boyd KL, Calabrese C, Stienstra R, van d, V, Netea MG, Joosten LA, Lamkanfi M *et al*: **Inflammasome-independent role of apoptosis-associated speck-like protein containing a CARD (ASC) in T cell priming is critical for collagen-induced arthritis**. *JBiolChem* 2010, **285**:12454-12462.

37. Joosten LA, Netea MG, Mylona E, Koenders MI, Malireddi RK, Oosting M, Stienstra R, van d, V, Stalenhoef AF, Giamarellos-Bourboulis EJ *et al*: **Engagement of fatty acids with Toll-like receptor 2 drives interleukin-1beta production via the ASC/caspase 1 pathway in monosodium urate monohydrate crystal-induced gouty arthritis**. *Arthritis Rheum* 2010, **62**:3237-3248.

38. Fang R, Tsuchiya K, Kawamura I, Shen Y, Hara H, Sakai S, Yamamoto T, Fernandes-Alnemri T, Yang R, Hernandez-Cuellar E *et al*: **Critical Roles of ASC Inflammasomes**

in Caspase-1 Activation and Host Innate Resistance to Streptococcus pneumoniae Infection. *JImmunol* 2011, **187**:4890-4899.

39. Ellebedy AH, Lupfer C, Ghoneim HE, DeBeauchamp J, Kanneganti TD, Webby RJ: **Inflammasome-independent role of the apoptosis-associated speck-like protein containing CARD (ASC) in the adjuvant effect of MF59**. *ProcNatlAcadSciUSA* 2011, **108**:2927-2932.

40. Taxman DJ, Holley-Guthrie EA, Huang MT, Moore CB, Bergstralh DT, Allen IC, Lei Y, Gris D, Ting JP: **The NLR adaptor ASC/PYCARD regulates DUSP10, mitogen-activated protein kinase (MAPK), and chemokine induction independent of the inflammasome**. *JBiolChem* 2011, **286**:19605-19616.

41. Ippagunta SK, Malireddi RK, Shaw PJ, Neale GA, Walle LV, Green DR, Fukui Y, Lamkanfi M, Kanneganti TD: **The inflammasome adaptor ASC regulates the function of adaptive immune cells by controlling Dock2-mediated Rac activation and actin polymerization**. *NatImmunol* 2011, **12**:1010-1016.

42. Taxman DJ, Zhang J, Champagne C, Bergstralh DT, Iocca HA, Lich JD, Ting JP: **Cutting edge: ASC mediates the induction of multiple cytokines by Porphyromonas gingivalis via caspase-1-dependent and -independent pathways**. *JImmunol* 2006, **177**:4252-4256.

43. Bolz DD, Sundsbak RS, Ma Y, Akira S, Kirschning CJ, Zachary JF, Weis JH, Weis JJ: **MyD88 plays a unique role in host defense but not arthritis development in Lyme disease**. *JImmunol* 2004, **173**:2003-2010.

44. Joosten LA, Koenders MI, Smeets RL, Heuvelmans-Jacobs M, Helsen MM, Takeda K, Akira S, Lubberts E, van de Loo FA, van den Berg WB: **Toll-like receptor 2 pathway drives streptococcal cell wall-induced joint inflammation: critical role of myeloid differentiation factor 88**. *JImmunol* 2003, **171**:6145-6153.

45. Liu-Bryan R, Scott P, Sydlaske A, Rose DM, Terkeltaub R: **Innate immunity conferred by Toll-like receptors 2 and 4 and myeloid differentiation factor 88 expression is pivotal to monosodium urate monohydrate crystal-induced inflammation**. *Arthritis Rheum* 2005, **52**:2936-2946.

46. Nigrovic PA, Mannion M, Prince FH, Zeft A, Rabinovich CE, van Rossum MA, Cortis E, Pardeo M, Miettunen PM, Janow G *et al*: **Anakinra as first-line disease-modifying therapy in systemic juvenile idiopathic arthritis: report of forty-six patients from an international multicenter series**. *Arthritis Rheum* 2011, **63**:545-555.

47. Tran AP, Edelman J: **Interleukin-1 inhibition by anakinra in refractory chronic tophaceous gout**. *IntJRheumDis* 2011, **14**:e33-e37.

Additional files

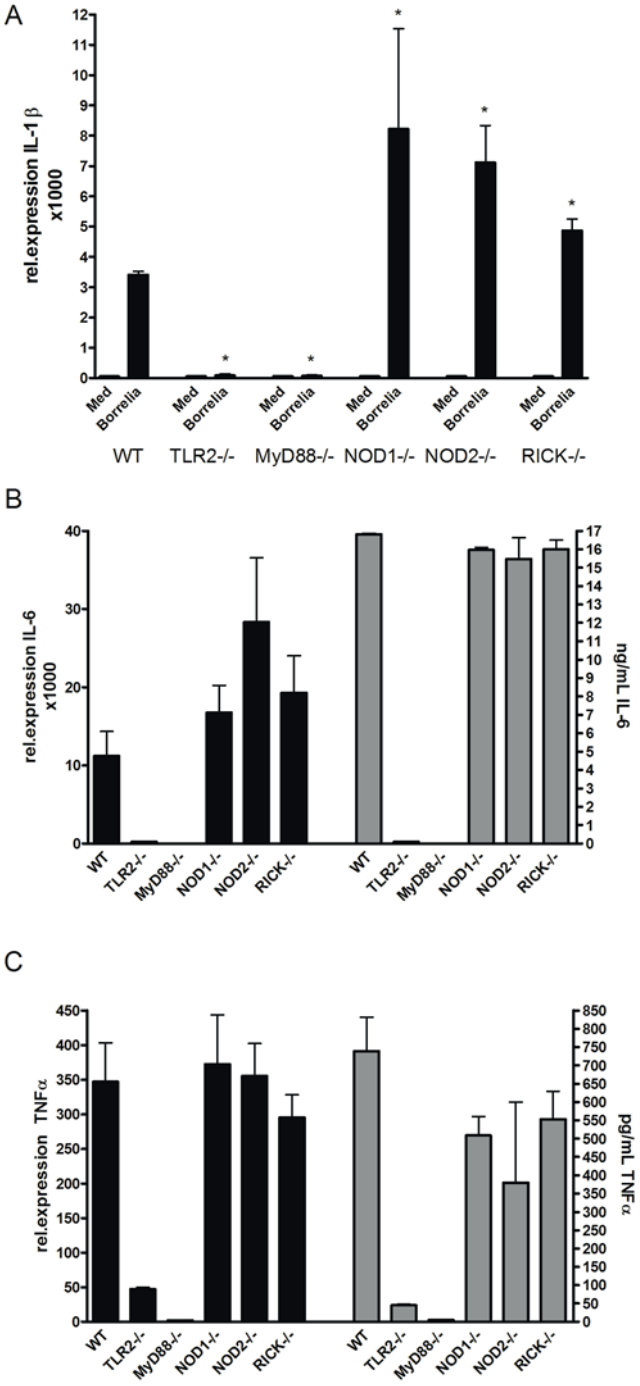


Figure S1. IL-1β mRNA expression is TLR2 and MyD88 dependent. (A) IL-1β mRNA expression levels (x1000) in bone marrow derived macrophages isolated from wild-type, TLR2-, MyD88-, NOD1-, NOD2-, and RICK-gene deficient mice. mRNA expression after 24 hours of stimulation with either medium or 5x10^6 B.burgdorferi per mL. At least 5 animals per group, bars represent mean±SEM. *p<0.05; Mann–Whitney U test, two-tailed. IL-6 (B) and TNF-α (C) mRNA expression and protein production (in ng/mL for IL-6, and pg/mL for TNF-α, respectively) by bone marrow derived macrophages isolated from wild-type, TLR2-, MyD88-, NOD1-, NOD2-, and RICK-gene deficient mice. mRNA expression after 24 hours of stimulation with either medium or 5x10^6 B.burgdorferi per mL. At least 5 animals per group, bars represent mean±SEM.

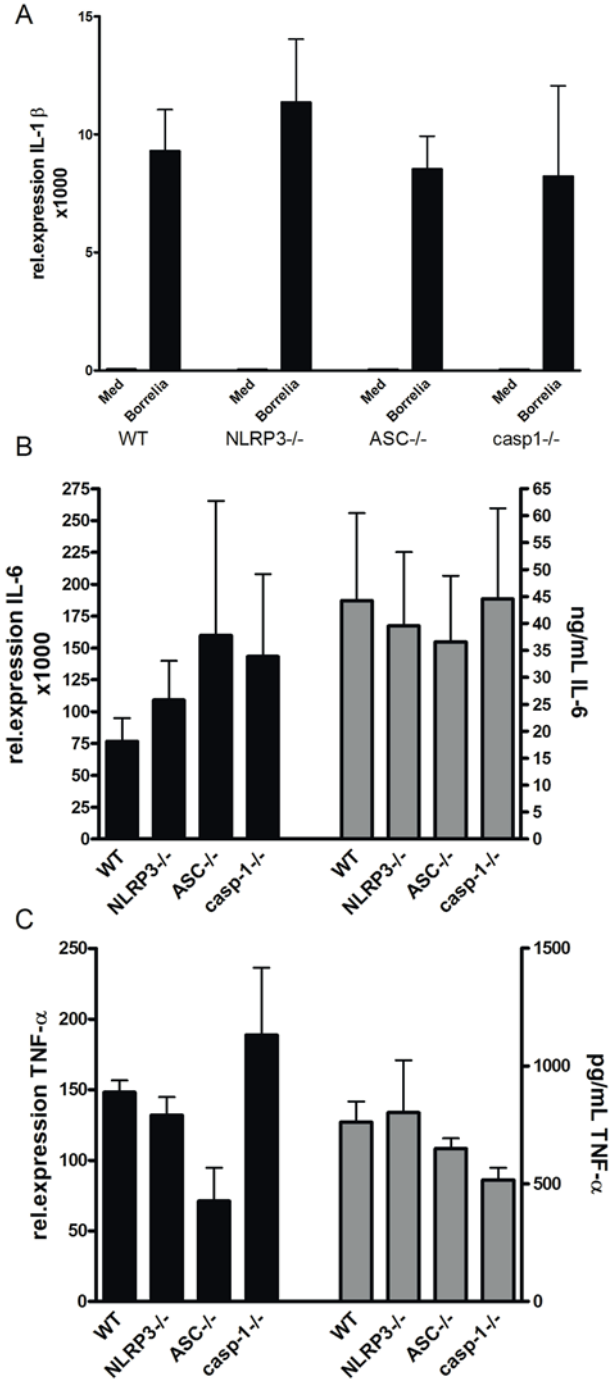


Figure S2. Inflammasome-independent IL-1β transcription. (A) IL-1β mRNA expression levels (x1000) in bone marrow derived macrophages isolated from wild-type, NLRP3-, ASC-, and caspase-1-gene deficient mice. mRNA expression after 24 hours of stimulation with either medium or 5x10^6 *B.burgdorferi* per mL. At least 5 animals per group, bars represent mean±SEM. IL-6 (B) and TNF-α (C) mRNA expression and protein production (in ng/mL for IL-6, and pg/mL for TNF-α, respectively) by bone marrow derived macrophages isolated from wild-type, NLRP3-, ASC-, and caspase-1-gene deficient mice. mRNA expression after 24 hours of stimulation with either medium or 5x10^6 *B.burgdorferi* per mL. At least 5 animals per group, bars represent mean±SEM.

Chapter 3

Autophagy modulates *Borrelia burgdorferi*-induced production of Interleukin-1β (IL-1β)

J Biol Chem. 2013 Mar 22;288(12):8658-66

Kathrin Buffen, Marije Oosting, Svenja Mennens, Paras K. Anand, Theo S. Plantinga, Patrick Sturm, Frank L. van de Veerdonk , Jos W.M. van der Meer, Ramnik J. Xavier, Thirumala-Devi Kanneganti, Mihai G. Netea and Leo A.B. Joosten

Summary

Borrelia burgdorferi sensu lato is the causative agent of Lyme disease (LD). Recent studies have shown that recognition of the spirochete is mediated by TLR2 and NOD2. The latter receptor has been associated with the induction of the intracellular degradation process called autophagy. The present study demonstrated for the first time the induction of autophagy by exposure to *B. burgdorferi* and that autophagy modulates the *B. burgdorferi*-dependent cytokine production. Human PBMCs treated with autophagy inhibitors showed an increased IL-1β and IL-6 production in response to the exposure of the spirochete, while TNFα production was unchanged. Autophagy induction against *B. burgdorferi* was dependent on reactive oxygen species (ROS) since cells from patients with chronic granulomatous disease (CGD), which are defective in ROS production, also produced elevated IL-1β. Further, the enhanced production of the pro-inflammatory cytokines was because of the elevated mRNA expression in the absence of autophagy. Our results thus demonstrate the induction of autophagy, which in-turn modulates cytokine production, by *B. burgdorferi* for the first time.

Introduction

Lyme disease, the most common vector-borne disease in the US and Western Europe, is caused by bacteria of the species *Borrelia burgdorferi sensu lato (1)*. Localized infection is typically manifested by an erythema migrans skin lesion. Depending on the subspecies, disseminated disease is mainly associated with arthritis (*B. burgdorferi*), skin disorders (*B. afzelii*), or neurological alterations (*B. garinii*) (2). Despite great advances in our understanding of the immunologic pathways triggered by the LD spirochete in humans (3-5), much remains to be learned regarding the immunopathogenesis of the disease. Several in vitro studies indicate the importance of lipid-modified proteins in the pathogenesis of the infection (6-8). The immune system recognizes these lipoproteins by different pattern recognition receptors (PRRs), of which Toll-like receptor 2 (TLR2) and Nucleotide-binding oligomerization domain-containing protein 2 (NOD2) are suggested to be the most important (4,9). The activation of PRRs after recognition of *Borrelia* results in the secretion of the pro-inflammatory cytokine IL-1β which is known to be involved in the pathogenesis of Lyme disease (10-12). IL-1β production requires two important steps: transcription of mRNA resulting in the production of proIL-1β protein, and cleavage of the immature precursor into mature bioactive IL-1β by the inflammasome-enzyme caspase-1 (13).

In addition to the induction of intracellular signals leading to the production of cytokines, engagement of PRRs such as NOD2 activates autophagy, a process in which damaged organelles or long-lived proteins are degraded (14-17). Autophagy involves the sequestration of dysfunctional proteins in a double-layered membrane called autophagosome, which is formed by the elongation of small membrane structures. The formation of this isolation membrane is initiated by autophagy-related gene (Atg) 16 and type III phosphatidylinositol 3-kinase (PI3K)(18). The delivery of dysfunctional proteins to the autophagosomes is regulated by autophagic adaptors such as p62. This latter protein can bind to the intracellular target as well as to the microtubule-associated protein 1 light chain 3 (LC3), which associates with the autophagosome after being processed (19). Autophagosomes mature through fusion with lysosomes, leading to the breakdown of the protein content (20).

The link between autophagy and the innate defense mechanism has been made in several studies describing the connection between dysfunctional autophagy and autoinflammatory diseases (21-24). It has been shown that the inhibition of autophagy

by chemical inhibitors of PI3 kinases leads to an enhancement of extracellular IL-1β after stimulation with bacterial wall components such as LPS (25). This observation, next to the fact that *B. burgdorferi* is thought to be recognized by the autophagy-inducing receptor NOD2, prompted us to investigate the role of autophagy in host defense during infection with *B. burgdorferi*. By stimulating human peripheral blood mononuclear cells (PBMCs), we demonstrate that inhibition of autophagy increases IL-1β and IL-6 production after stimulation with *Borrelia* bacteria. The enhanced production was specific for IL-1β and IL-6, while TNFα production was unchanged. The robust increase in mRNA synthesis of the proinflammatory cytokines IL-1β and IL-6 indicated that autophagy regulates *Borrelia*-induced IL-1β production at the transcriptional level.

Experimental Procedures

Borrelia burgdorferi cultures. *B. burgdorferi,* ATCC strain 35210, was cultured at 33°C in Barbour-Stoenner-Kelley (BSK)-H medium (Sigma-Aldrich) supplemented with 6% rabbit serum. Spirochetes were grown to late-logarithmic phase and examined for motility by dark-field microscopy. Organisms were quantitated by fluorescence microscopy after mixing 10 µL aliquots of culture material with 10 µL of an acridine orange solution and counted using a Petroff-Hauser counting chamber. Bacteria were harvested by centrifugation of the culture at 7000 x g for 15 min., washed twice with sterile PBS (pH 7.4), and diluted in the specified medium to required concentrations.

Isolation of human peripheral blood mononuclear cells and *in vitro* cytokine production. Venous blood was drawn from the cubital vein of healthy volunteers or patients with CGD into 10 ml EDTA tubes (Monoject, Covidien, Mansfield, Massachusetts, USA). The mononuclear cell fraction was obtained by density centrifugation of blood diluted 1:1 in pyrogen-free saline over Ficoll-Paque (Pharmacia Biotech, Pittsburgh, Pennsylvania, USA). Cells were washed twice in saline and suspended in culture medium (RPMI; Invitrogen, Carlsbad, California, USA) supplemented with gentamicin 10 mg/ml, L-glutamine 10 mM and pyruvate 10 mM. Cells were counted in a Coulter counter (Coulter Electronics, Brea, California, USA) and the number was adjusted to $5x10^6$ cells/ ml. A total of $5x10^5$ mononuclear cells in a 100 ml volume was added to round-bottom 96-well plates (Greiner, Monroe, North Carolina, USA) and incubated with either 100 ml of culture medium (negative control), or *B. burgdorferi* (MOI 0.2). In some experiments, PBMCs

were pre-incubated with culture medium or the autophagy inhibitors 3-methyl adenine (3-MA, 10 mM; Sigma), Wortmannin (10 µg/mL, BioLegend) or LY294002 (100µM, Invivogen) for 60 minutes. After 24 hours, supernatants were collected and stored at −20°C until being assayed.

Real-time PCR. RNA from PBMCs was isolated using TRIzol reagent (Invitrogen) following the manufacturer's instructions. Isolated RNA was reversed transcribed into complementary DNA using iScript cDNA synthesis kit (Bio-Rad Laboratories BV, Veenendaal, the Netherlands). Quantitative real-time PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems) using a 7300 Real-time PCR system (Applied Biosystems). In each PCR reaction a melting curve analysis was included to control for aspecific PCR amplification. Primers used for the experiments (final concentration 10µM) are shown below. Real-time qPCR data were corrected for expression of the housekeeping gene human *GAPDH*. Human *IL-1β*; Forward sequence 5'-3': GCC-CTA-AAC-AGA-TGA-AGT-GCT-C, Reversed sequence 5'-3': GAA-CCA-GCA-TCT-TCC-TCA-G, Human *IL-6*; Forward sequence 5'-3': AAT-TCG-GTA-CAT-CCT-CGA-CGG, Reversed sequence 5'-3': GGT-TGT-TTT-CTG-CCA-GTG-CCT, Human *TNFα*; Forward sequence 5'-3': TGG-CCC-AGG-CAG-TCA-GA, Reversed sequence 5'-3': GGT-TTG-CTA-CAA-CAT-GGG-CTA-CA, Human *GAPDH*; Forward sequence 5'-3': AGG-GGA-GAT-TCA-GTG-TGG-TG, Reversed sequence 5'-3': CGA-CCA-CTT-TGT-CAA-GCT-CA. Cycling conditions were 2 min at 50°C and 10 min at 95°C followed by 40 cycli of 95°C for 15 sec and 1 min at 60°C.

Cytokine measurements. Concentrations of human IL-1β, pro-IL-1β IL-6, TNFα, or IL-1RA were determined in duplicates using specific commercial ELISA kits (Sanquin, Amsterdam, or R&D Systems, Minneapolis), in accordance with the manufacturers' instructions. Levels of bioactive IL-1 were measured using a murine thyoma cell line EL4/NOB1 that produces IL-2 in response to active IL-1 as described above.

Western blot. For Western blotting of LC3 and GAPDH, cells were washed and samples prepared for SDS-PAGE by lysing the cells in RIPA buffer. After protein electrophoresis in a 15% polyacrylamide gel, proteins were transferred to a nitrocellulose membrane by western blotting. The membrane was first incubated with rabbit primary antibody specific for LC3-II or GAPDH, followed by a secondary goat-anti-rabbit antibody conjugated to horseradish peroxidase. Enhance chemiluminescence was used to detect the proteins. For Western blotting of caspase-1 and actin, cells were washed twice with sterile PBS

and lysed in buffer (150 mM NaCl, 10 mM Tris, pH 7.4, 5 mM EDTA, 1 mM EGTA, 0.1% Nonidet P-40) supplemented with a Roche protease inhibitor cocktail tablet. Proteins were separated using SDS-PAGE (12% polyacrylamide gel) and subsequently transferred to a PVDF membrane. The membranes were coated with primary antibodies and active caspase-1 or actin were detected using secondary anti-rabbit antibody conjugated to horseradish peroxidase followed by enhanced chemiluminescence.

Fluorescence Microscopy. HeLa cells were transfected with a plasmid containing GFP-LC3 (kindly provided by Dr. T Yoshimori, Osaka, Japan) using the transfection medium Fugene 6 (Roche) according to the manufacturer's instructions. GFP-LC3+ HeLa cells were grown and stimulated on coverslips (19-mm diameter) in 12-well plates. Cells were fixed with 2% paraformaldehyde for 15 min at room temperature and permeabilized for 10 min with cold methanol (100%). After washing with PBS (×3), the coverslips were mounted onto glass slides with Vectashild +DAPI and analyzed on a fluorescence microscope.

Confocal microscopy. HeLa cells were grown and stimulated on coverslips (19-mm diameter) in 12-well plates. Cells were fixed with 2% paraformaldehyde for 15 min at room temperature. After washing with PBS, cells were stained with anti HLA class I Alexa Fluor 649 + DAPI and analyzed on a confocal microscope.

ROS measurement. PBMCs were suspended in HBSS (Hank's Buffered Salt Solution) and exposed to different concentrations of *B. burgdorferi*. ROS formation was measured by a chemiluminescence assay using luminol (5mM, 5-amino-2,3-dihydro-1,4-phthalazinedione, Sigma). The luminometer measured chemiluminescence in the integration mode at 37°C for 1 hour after luminol had been added.

Ethics statement. All human experiments were conducted according to the principles expressed in the Declaration of Helsinki. Before taking blood, informed written consent of each human subject was provided. The study was approved by the review board of Radboud University Nijmegen Medical Centre.

Statistical Analysis. Data are expressed as mean ± SEM unless otherwise indicated. Differences between experimental groups were tested using the two-sided Mann-Whitney *U* test performed on GraphPad Prism 4.0 software (GraphPad). *P* values of ≤0.05 were considered significant.

Results

***B. burgdorferi* induces autophagy.** Previous studies demonstrated that autophagy inhibits LPS-induced IL-1β in human PBMCs (25). Since IL-1β is associated with the pathogenesis of Lyme disease (12), we assessed the role of autophagy during the exposure to *B. burgdorferi* based on microtubule associated protein 1 light chain 3 (LC3). During autophagy, the cytoplasmic form (LC3-I) is processed into the phosphatidylethanolamine-conjugated form (LC3-II), the latter indicating activation of autophagy inside the cells. GFP-LC3 expressing HeLa cells were exposed to *B. burgdorferi* alone or in combination with the autophagy inhibitors 3-Methyl Adenine (3MA) or Wortmannin (Figure 1A). An increase in autophagosome formation can be seen after exposure to *B. burgdorferi* demonstrated by a high number of GFP-LC3+ punctae. 3MA and Wortmannin inhibited the formation of those vesicles attesting the validity of this model. Previously, it has been demonstrated that primary cells as PBMCs but not all cell lines are able to phagocytose the spirochetes (26). Therefore, the ability of the used model (HeLa cells) to internalize *B. burgdorferi* has been investigated. Internalized spirochetes can be seen indicating the legality of the model (Figure 1B). Western blot analysis of LC3 in murine BMDMs showed an increased amount of LC3-II after exposure to *B. burgdorferi* (Figure 1C).

Inhibition of autophagy enhances IL-1β and IL-6 production but not TNFα. To determine the role of autophagy in the induction of proinflammatory cytokines by *B. burgdorferi*, PBMCs obtained from healthy volunteers were stimulated for 24h with the spirochete alone or in combination with different autophagy inhibitors. 3MA blocks the activity of PI3-kinases, which are mandatory for the initiation of autophagy. PBMCs treated with autophagy inhibitors showed significantly higher IL-1β and IL-6 production after exposure to *B. burgdorferi* (Figure 2A/B). To corroborate the findings of 3MA, other inhibitors were used (Wortmannin, LY294002). In contrast, no increase in TNFα production could be observed by blocking autophagy with 3MA, Wortmannin or LY294003 (Figure 2C). In line with the results of the secreted IL-1β protein, intracellular concentrations of total IL-1β were also enhanced (Figure 2G). To examine whether inhibition of autophagy during exposure to *Borrelia* results in altered mRNA levels, we measured transcription of several cytokines. IL-1β and IL-6 mRNA levels were strongly increased in human PBMCs after *Borrelia* recognition when autophagy was inhibited (Figure 2D+E). In contrast, the level of TNFα mRNA was not influenced (Figure 2F). To exclude any alteration of cytokine production due to the autophagy inhibitors alone, we blocked autophagy in

non-stimulated PBMCs, and no increase of cytokine production was observed (data not shown). Using murine ATG7 deficient bone marrow derived macrophages (BMDM), an enhanced production of IL-1β could be seen after the exposure to *B. burgdorferi* (data not shown). These finding underscore our results that inhibition of autophagy leads to enhanced IL-1β production after *Borrelia* exposure.

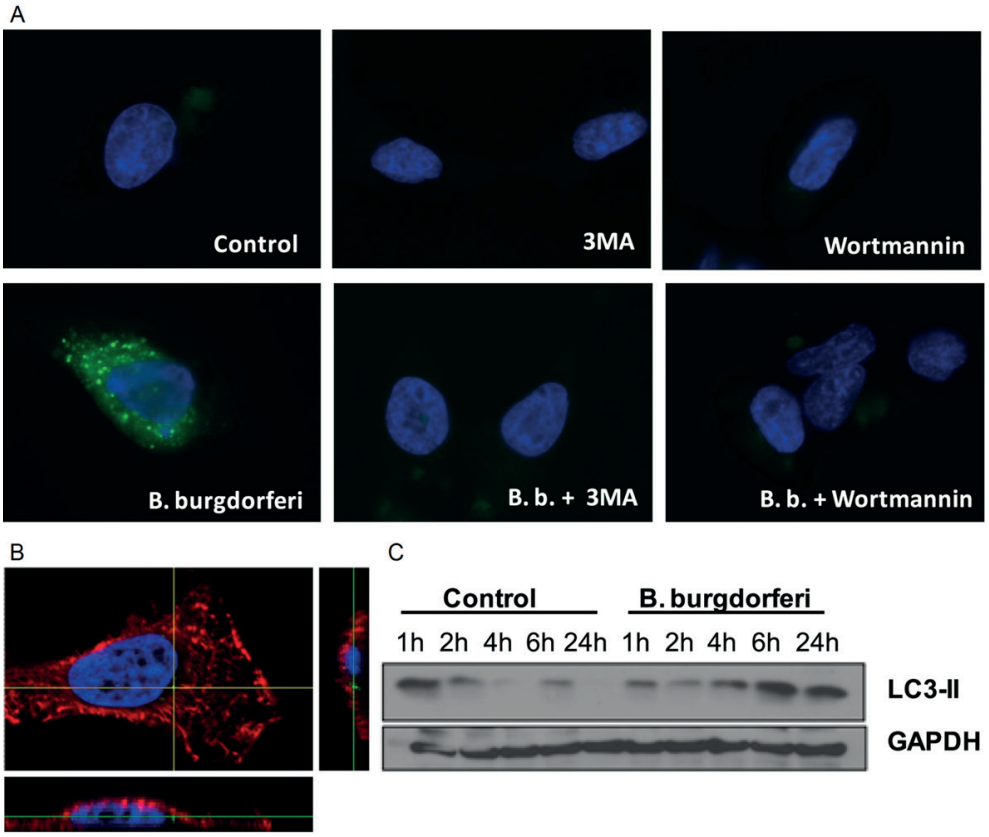


FIGURE 1: Activation of autophagy by *Borrelia burgdorferi*. (A) GFP-LC3-expressing HeLa cells were pre-incubated for 1 hour at 37°C in RPMI alone, containing 3MA (10mM) or Wortmannin (100nM) in which inhibitors of lysosomal fusion such as Ammonium chloride (20mM) and Leupeptine (100mM) have been added. After 2 hours of stimulation with culture medium or *B. burgdorferi* (MOI 0.4), cells were fixed, nuclei stained with DAPI (blue) and slides were analyzed by fluorescence microscopy. Data are representative of at least four experiments. (B) HeLa cells were exposed to GFP- labeled *B. burgdorferi* (MOI 1) for 2 hours, cells were fixed, membrane stained with anti HLA class I Alexa Fluor 649 (red) and nuclei stained with DAPI (blue). Slides were analyzed by confocal microscopy. Data are representative of at least four experiments (C) Western Blot analysis of LC3-II in lysates of mouse BMDMs stimulated with *B. burgdorferi* (MOI 5) for indicated periods of time.

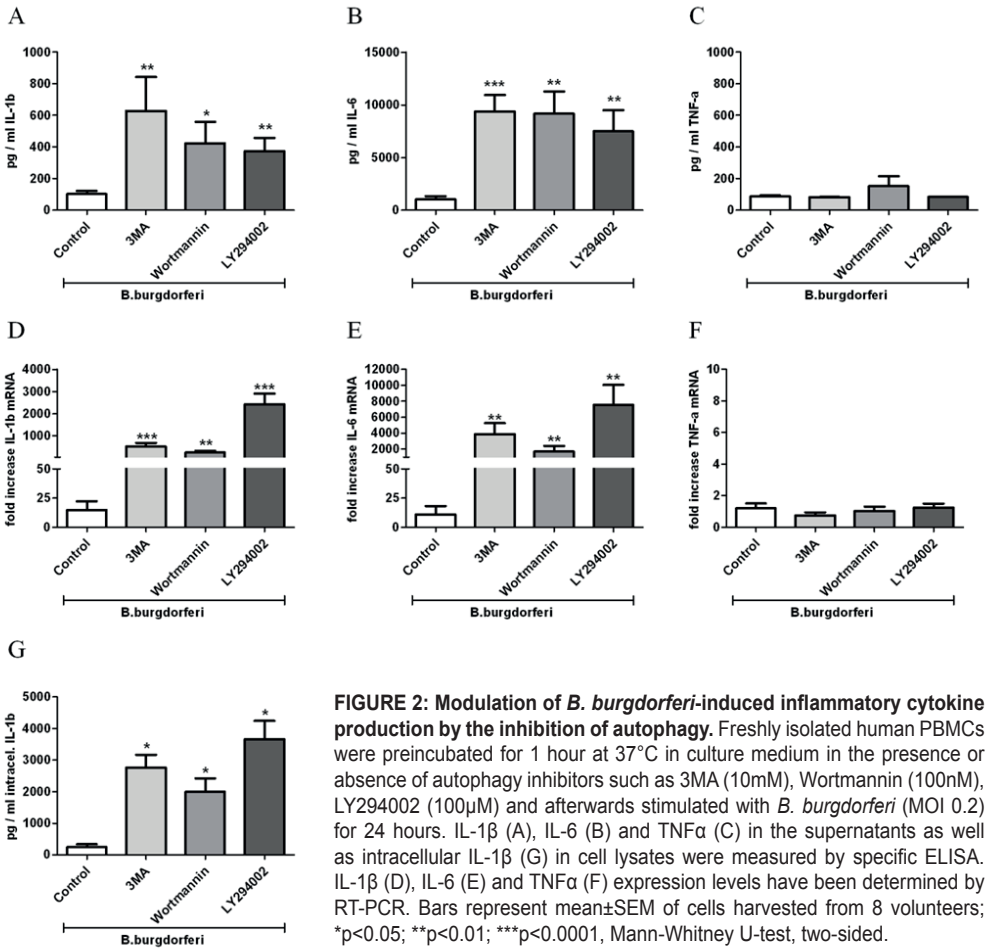


FIGURE 2: Modulation of *B. burgdorferi*-induced inflammatory cytokine production by the inhibition of autophagy. Freshly isolated human PBMCs were preincubated for 1 hour at 37°C in culture medium in the presence or absence of autophagy inhibitors such as 3MA (10mM), Wortmannin (100nM), LY294002 (100μM) and afterwards stimulated with *B. burgdorferi* (MOI 0.2) for 24 hours. IL-1β (A), IL-6 (B) and TNFα (C) in the supernatants as well as intracellular IL-1β (G) in cell lysates were measured by specific ELISA. IL-1β (D), IL-6 (E) and TNFα (F) expression levels have been determined by RT-PCR. Bars represent mean±SEM of cells harvested from 8 volunteers; *p<0.05; **p<0.01; ***p<0.0001, Mann-Whitney U-test, two-sided.

Increased extracellular IL-1β is not based on the secretion of immature IL-1β protein. To exclude the possibility of an increased secretion of immature (and inactive) IL-1β by autophagy inhibitors, the bioactivity of extracellular IL-1β was measured using a murine thyoma cell line EL4/NOB1, which produces IL-2 in response to active IL-1. The amount of bioactive IL-1 increased in the presence of autophagy inhibitors (Figure 3A). To support these data, the amount of pro-IL-1β was measured in the supernatants of PBMCs incubated with *B. burgdorferi* alone or in combination with autophagy inhibitors. No difference in the amount of proIL-1β could be found in the supernatant of the various samples (Figure 3B). An increase in proinflammatory cytokines due to cell death was also excluded, since the level of LDH, a marker for cell death, stayed below the detection level in all samples (data not shown).

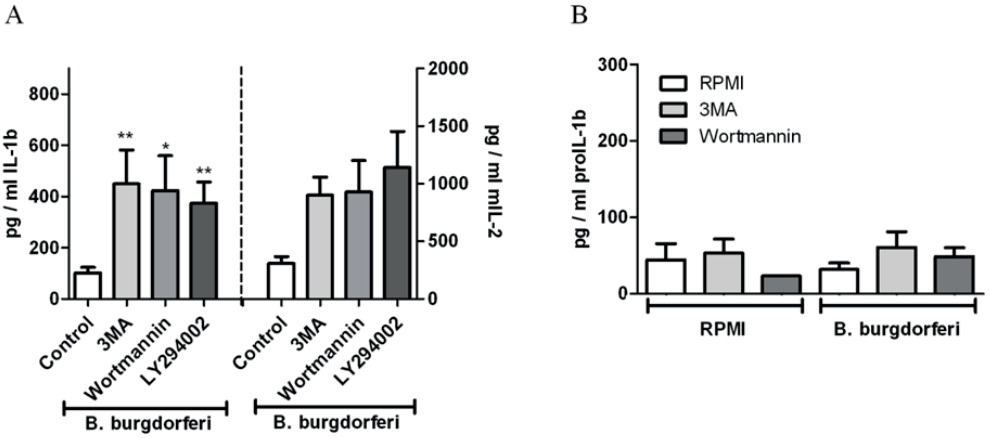


FIGURE 3: Bioactivity of extracellular IL-1β. Freshly isolated human PBMCs were preincubated for 1 hour at 37°C in culture medium in the presence or absence of autophagy inhibitors such as 3MA (10mM), Wortmannin (100nM), LY294002 (100μM) and afterwards stimulated with *B. burgdorferi* (MOI 0.2) for 24 hours. (A) Supernatant was added to murine NOB-1 cells which react to functional human IL-1 with the secretion of IL-2. Murine IL-2 was measured in the supernatant of NOB-1 cells after 24h. (B) Pro-IL-1β was measured in supernatants using a specific ELISA. Data from 4 volunteers are shown as mean±SEM; *p<0.05; **p<0.01, Mann-Whitney U-test, two-sided.

Inhibition of autophagy does not increase caspase-1 activity. IL-1β is synthesized as an immature pro-form (pro-IL-1β) which requires activated caspase-1, a component of the inflammasome, for cleavage into mature IL-1β secreted from the cells. To determine the role of autophagy in the activation of caspase-1 leading to processing of immature IL-1β, PBMCs obtained from healthy volunteers were stimulated with *B. burgdorferi* alone or in combination with several autophagy inhibitors for 3h. The amount of active caspase-1 stayed the same independent of the inhibition of autophagy (Figure 4). Furthermore, the influence of caspase-1 on the induction of autophagy by *B. burgdorferi* has been analyzed using GFP-LC3 expressing HeLa cells incubated with the caspase-1 inhibitor YVAD and *Borrelia*. No effect on autophagosome formation by caspase-1 inhibition was seen after exposure of the GFP-LC3 cells to *B. burgdorferi* (data not shown).

***B. burgdorferi*-induced reactive oxygen species (ROS) reduce the production of IL-1β.** It has been proposed that the formation of reactive oxygen species forms a necessary step in the induction of autophagy (27,28). We therefore hypothesized that defects in the production of ROS would result in a defective autophagic process, followed by an increase in IL-1β production. The exposure of *B. burgdorferi* to PBMCs resulted in a significant increase in ROS compared to unstimulated samples (Figure 5A). To further assess the production of cytokines in response to ROS, PBMCs of patients with chronic

granulomatous disease (CGD), who have a defective production of reactive oxygen species, were exposed to *B. burgdorferi* for 24h (Figure 5B). Cells of CGD patients showed higher production of IL-1β compared to healthy controls, supporting our previous data of increased *B. burgdorferi*-induced cytokines during impaired autophagy (Figure 2A).

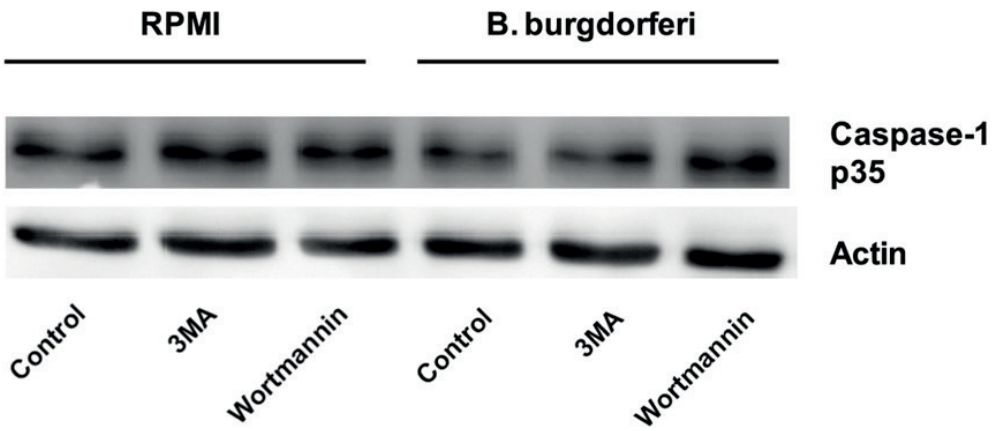


FIGURE 4: Autophagy does not influence the activation of caspase-1. Western Blot analysis of caspase-1 in lysates of autophagy-impaired human PBMCs stimulated with *B. burgdorferi* (MOI 2). PBMCs were pre-incubated for 1 hour at 37°C in RPMI alone, containing 3MA (10mM) or Wortmannin (100nM). After 3 hours of stimulation with culture medium or *B. burgdorferi*, cells have been lysed and were further analyzed with Western Blot. The picture is representative for results obtained from 4 volunteers.

Enhanced production of IL-1β and IL-6 during inhibition of autophagy is not based on IL-1β feedback-loop. Previous studies showed that exogenous IL-1β induces endogenous IL-1β and IL-6 mRNA expression and protein production. To assess the influence of extracellular bioactive IL-1β on the production of cytokines by PBMCs, which have been treated with autophagy inhibitors, we blocked the IL-1 receptor using IL-1ra (Anakinra) and analyzed the production of cytokines at mRNA level as well as secreted protein. PBMCs treated with IL-1ra in combination with 3MA or Wortmannin produce a larger amount of IL-1β and IL-6 in response to *B. burgdorferi* at both mRNA expression and protein levels (Figure 6 A, B, D, E, G). In contrast, TNFα mRNA expression, as well as the secreted TNFα protein, was not affected by autophagy inhibitors even in the presence

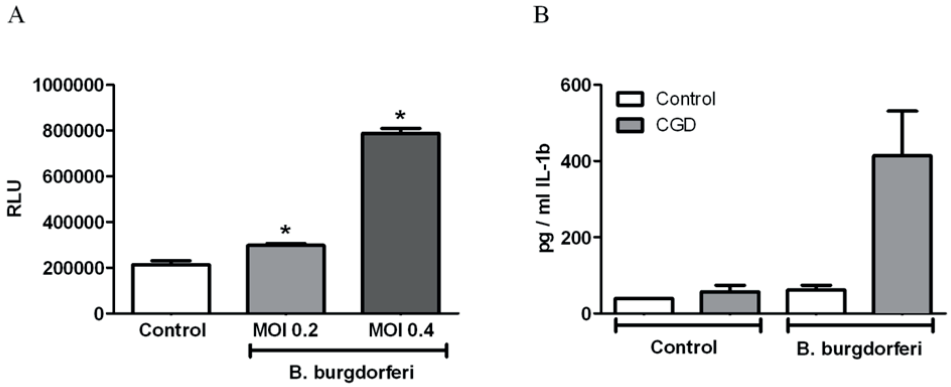


FIGURE 5: Production of IL-1β after *B. burgdorferi* stimulation is controlled by Reactive Oxygen Species. (A) Freshly isolated human PBMCs were stimulated with *B. burgdorferi* for one hour. The production of ROS was measured by chemiluminescence using luminol. (B) Freshly isolated human PBMCs of healthy volunteers and CGD patients were stimulated with *B. burgdorferi* for 24 hours. IL-1β was measured in the supernatant by specific ELISA. Bars represent mean±SEM; *p<0.05, Mann-Whitney U-test, two-sided. N=4 controls, patients.

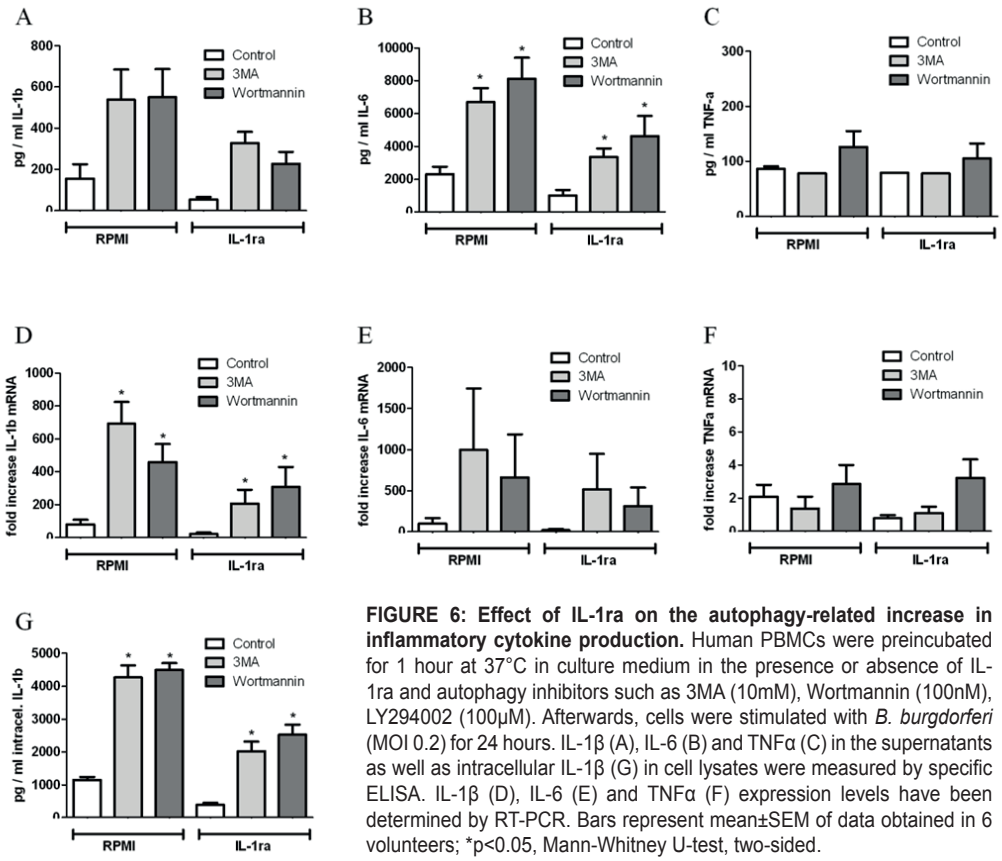


FIGURE 6: Effect of IL-1ra on the autophagy-related increase in inflammatory cytokine production. Human PBMCs were preincubated for 1 hour at 37°C in culture medium in the presence or absence of IL-1ra and autophagy inhibitors such as 3MA (10mM), Wortmannin (100nM), LY294002 (100μM). Afterwards, cells were stimulated with *B. burgdorferi* (MOI 0.2) for 24 hours. IL-1β (A), IL-6 (B) and TNFα (C) in the supernatants as well as intracellular IL-1β (G) in cell lysates were measured by specific ELISA. IL-1β (D), IL-6 (E) and TNFα (F) expression levels have been determined by RT-PCR. Bars represent mean±SEM of data obtained in 6 volunteers; *p<0.05, Mann-Whitney U-test, two-sided.

Discussion

The link between autophagy and innate immunity, and its role as a defense mechanism against invading pathogens has been made in several studies (29-31), suggesting a regulatory role of autophagy on inflammasome activation and production of cytokines upon stimulation with microbial ligands (32). Since IL-1β has been demonstrated to contribute to the pathogenesis of Lyme disease (12), we examined the role of autophagy for the production of this pivotal proinflammatory cytokine in response to *B. burgdorferi*. Although both mRNA expression and production of TNFα was not enhanced in autophagy-blocked PBMCs stimulated with the *Borrelia*-spirochete, secretion of IL-1β and IL-6 protein was highly elevated. In addition, the mRNA synthesis of IL-1 β and IL-6 was strongly increased as well, indicating that the inhibition of autophagy modulates IL-1β and IL-6 production at the transcriptional level.

IL-1β is synthesized as an inactive pro-cytokine (pro-IL-1β) and requires activated caspase-1, a component of the inflammasomes, for cleavage into mature bioactive IL-1β. Since recent studies revealed an association between genetic disorders involving the activation of the inflammasome and autoinflammatory disorders (33-35), we investigated the effect of autophagy on the activation of inflammasome/caspase-1. In contrast to murine studies (36), autophagy did not inhibit inflammasome activation in human PBMCs, supporting our previous studies that demonstrated that autophagy inhibition affected the transcription, rather than processing, of IL-1β. The constitutive active caspase-1 in primary human monocytes, in contrast to the inactive caspase-1 of mouse macrophages, may explain the different results (37). An increased release of pro-IL-1β secretion due to cell death could be excluded by evaluation of the bioactivity of IL1 that was significantly increased by autophagy inhibitors (Fig 3).

The increased production of IL-6 after inhibition of autophagy is most probably a consequence of increased bioactive IL-1β, since it has been shown that IL-1receptor signaling is a major inducer of IL-6 (38). Interestingly, production of TNFα was not affected, which can be explained by different induction pathways. It has been shown that p38-MAPK and NF-κB are main inducers of TNFα transcription, whereas additional pathways such as extracellular signal-related kinase/nuclear factor kappa B (Erk/NF-κB) are needed for the transcription of IL-1β (39,40).

Recent studies have revealed that formation of reactive oxygen species (ROS) forms a necessary step in the initiation of autophagy (27,28). Several TLRs are able to activate Nox2 leading to the generation of ROS. These oxidative conditions are essential for autophagy induction, as treatment with anti-oxidative agents abolishes the formation of autophagosomes (27). We therefore hypothesized that defects in the production of ROS would result in a defective autophagic process, followed by an increase in IL-1β production. Consistent with this hypothesis, cells isolated from patients with CGD, who have a defective production of ROS due to genetic defects in the genes forming the NADPH complex, had an enhanced production of IL-1β compared to healthy controls (Fig 5). These additional data support our results of increased production of *B. burgdorferi*-induced cytokines in autophagy-impaired cells (Figure 2A/B).

Extracellular IL-1β performs its pro-inflammatory effect via an autocrine mechanism followed by binding to its cognate receptor IL1 receptor type I (41). In the present study, we used recombinant IL-1 receptor antagonist (IL-1ra) to determine the contribution of an IL-1-positive feedback loop to the total amount of *B. burgdorferi*-induced cytokine production. IL-1R blockade in autophagy capable cells resulted in reduced cytokine concentrations after *Borrelia* exposure. However, in cells in which autophagy was impaired, we noted an increased induction of cytokines by *B. burgdorferi* even if IL-1ra was added to the medium to block the IL1-induced loop, suggesting an IL-1R independent mechanism.

In previous reports it was described that autophagy plays a role in the cytokine response to several TLR-ligands (32,42). However, this is the first study to show an influence of autophagy on the *Borrelia*-induced cytokine response. The precise mechanism underlying the effect of autophagy on IL-1β production induced by *B. burgdorferi* remains to be elucidated in further detail. Lee et al. (43) proposed a model in which autophagy suppresses the IL-1β signaling by downregulating p62 levels (25,43). P62 acts a selective autophagy receptor for ubiquitinated protein aggregates (44) and as an important scaffold in the IL-1β signaling pathway by promoting oligomerization and activation of TRAF6 resulting in the transcription of NF-κB (45) and consequently increased IL-1β production. The expression level of p62 is important for these functions and it is thought to be regulated by autophagy. In the absence of this degradation process, increased p62 levels promote oligomerization and activation of TRAF6 resulting in overactivation of NF-κB. Therefore, we propose that the recognition of *B. burgdorferi* by TLR2 and NOD2 induces the production of IL-1β. In the absence of autophagy, p62 levels will be elevated, leading

to even more TRAF6 aggregation and consequently more IL-1β production.

The autophagy-dependent increase of IL-1β in PBMCs seems to be *Borrelia*-specific, since the exposure of different pathogens such as *Candida albicans* to autophagy-impaired cells resulted in a decreased IL-1β response (data not shown). Therefore, a better understanding of the precise mechanisms underlying the altered IL-1β production upon *B. burgdorferi* stimulation in autophagy-impaired cells may lead to the identification of novel therapeutic targets that may be important in the pathogenesis of Lyme disease in which IL1-dependent inflammation is central.

It is tempting to speculate that inhibition of autophagy could be beneficial during the early phase of Lyme disease leading to higher concentrations of IL-1β and therefore a possibly improved clearance of the pathogen. However, since Lyme disease occurs in different stadia, enhanced IL-1β production by autophagy modulation in disseminated Lyme disease might be detrimental for the disease activity. Several studies indicated a link between a high concentrations of IL-1β and pathogenic Th17 cells l leading to increased joint damage in rheumatoid arthritis (RA) or psoriasis patients (46,47). Therefore, the induction of autophagy in the disseminated state of Lyme disease could be favorable for Lyme patients through a decreased IL-1β production and inhibition of pathogenic Th17 cells.

In summary, we have elucidated the link between *B. burgdorferi* recognition and an important regulatory mechanism of inflammation by autophagy in Lyme disease. These findings may reveal novel therapeutic targets to treat Lyme disease patients in the future.

Acknowledgments

We thank Carla Bartels (Department of Medical Microbiology, Radboud University Nijmegen Medical Centre) for culturing and counting *Borrelia* spirochetes. We would also like to thank Dr. T Yoshimori (Osaka, Japan) for providing the plasmid containing GFP-LC3 and Huib Croes for his help using the confocal microscope.

References

1. Stanek, G., Wormser, G. P., Gray, J., and Strle, F. (2012) Lyme borreliosis. *Lancet* **379**, 461-473

2. Derdakova, M., and Lencakova, D. (2005) Association of genetic variability within the Borrelia burgdorferi sensu lato with the ecology, epidemiology of Lyme borreliosis in Europe. *Annals of agricultural and environmental medicine : AAEM* **12**, 165-172

3. Cruz, A. R., Moore, M. W., La Vake, C. J., Eggers, C. H., Salazar, J. C., and Radolf, J. D. (2008) Phagocytosis of Borrelia burgdorferi, the Lyme disease spirochete, potentiates innate immune activation and induces apoptosis in human monocytes. *Infect Immun* **76**, 56-70

4. Oosting, M., Ter Hofstede, H., Sturm, P., Adema, G. J., Kullberg, B. J., van der Meer, J. W., Netea, M. G., and Joosten, L. A. (2011) TLR1/TLR2 heterodimers play an important role in the recognition of Borrelia spirochetes. *PLoS One* **6**, e25998

5. Cervantes, J. L., Dunham-Ems, S. M., La Vake, C. J., Petzke, M. M., Sahay, B., Sellati, T. J., Radolf, J. D., and Salazar, J. C. (2011) Phagosomal signaling by Borrelia burgdorferi in human monocytes involves Toll-like receptor (TLR) 2 and TLR8 cooperativity and TLR8-mediated induction of IFN-beta. *Proc Natl Acad Sci U S A* **108**, 3683-3688

6. Vincent, M. S., Roessner, K., Sellati, T., Huston, C. D., Sigal, L. H., Behar, S. M., Radolf, J. D., and Budd, R. C. (1998) Lyme arthritis synovial gamma delta T cells respond to Borrelia burgdorferi lipoproteins and lipidated hexapeptides. *J Immunol* **161**, 5762-5771

7. Hefty, P. S., Jolliff, S. E., Caimano, M. J., Wikel, S. K., Radolf, J. D., and Akins, D. R. (2001) Regulation of OspE-related, OspF-related, and Elp lipoproteins of Borrelia burgdorferi strain 297 by mammalian host-specific signals. *Infect Immun* **69**, 3618-3627

8. Norgard, M. V., Arndt, L. L., Akins, D. R., Curetty, L. L., Harrich, D. A., and Radolf, J. D. (1996) Activation of human monocytic cells by Treponema pallidum and Borrelia burgdorferi lipoproteins and synthetic lipopeptides proceeds via a pathway distinct from that of lipopolysaccharide but involves the transcriptional activator NF-kappa B. *Infect Immun* **64**, 3845-3852

9. Oosting, M., Berende, A., Sturm, P., Ter Hofstede, H. J., de Jong, D. J., Kanneganti, T. D., van der Meer, J. W., Kullberg, B. J., Netea, M. G., and Joosten, L. A. (2010) Recognition of Borrelia burgdorferi by NOD2 is central for the induction of an inflammatory reaction. *J Infect Dis* **201**, 1849-1858

10. Liu, N., Belperron, A. A., Booth, C. J., and Bockenstedt, L. K. (2009) The caspase 1 inflammasome is not required for control of murine Lyme borreliosis. *Infect Immun* **77**, 3320-3327

11. Petzke, M. M., Brooks, A., Krupna, M. A., Mordue, D., and Schwartz, I. (2009) Recognition of Borrelia burgdorferi, the Lyme disease spirochete, by TLR7 and TLR9 induces a type I IFN response by human immune cells. *J Immunol* **183**, 5279-5292

12. Pietruczuk, A., Swierzbinska, R., Pietruczuk,

M., and Hermanowska-Szpakowicz, T. (2004) [Role of interleukin-18, interleukin-1beta and its soluble receptor (sIL-1RII) in early and late Lyme borreliosis]. *Pol Merkur Lekarski* **17**, 446-450

13. Dinarello, C. A. (2009) Immunological and Inflammatory Functions of the Interleukin-1 Family. *Annu Rev Immunol* **27**, 519-550

14. Cooney, R., Baker, J., Brain, O., Danis, B., Pichulik, T., Allan, P., Ferguson, D. J., Campbell, B. J., Jewell, D., and Simmons, A. (2010) NOD2 stimulation induces autophagy in dendritic cells influencing bacterial handling and antigen presentation. *Nat Med* **16**, 90-97

15. Plantinga, T. S., Joosten, L. A., and Netea, M. G. (2011) ATG16L1 polymorphisms are associated with NOD2-induced hyperinflammation. *Autophagy* **7**, 1074-1075

16. Netea, M. G., and Joosten, L. A. (2010) A NOD for autophagy. *Nat Med* **16**, 28-30

17. Anand, P. K., Tait, S. W., Lamkanfi, M., Amer, A. O., Nunez, G., Pages, G., Pouyssegur, J., McGargill, M. A., Green, D. R., and Kanneganti, T. D. (2011) TLR2 and RIP2 pathways mediate autophagy of Listeria monocytogenes via extracellular signal-regulated kinase (ERK) activation. *J Biol Chem* **286**, 42981-42991

18. Oh, J. E., and Lee, H. K. (2012) Modulation of pathogen recognition by autophagy. *Front Immunol* **3**, 44

19. Ponpuak, M., Davis, A. S., Roberts, E. A., Delgado, M. A., Dinkins, C., Zhao, Z. J., Virgin, H. W., Kyei, G. B., Johansen, T., Vergne, I., and Deretic, V. (2010) Delivery of Cytosolic Components by Autophagic Adaptor Protein p62 Endows Autophagosomes with Unique Antimicrobial Properties. *Immunity* **32**, 329-341

20. Kundu, M., and Thompson, C. B. (2008) Autophagy: Basic principles and relevance to disease. *Annu Rev Pathol-Mech* **3**, 427-455

21. Marquez, A., Nunez, C., Martinez, A., Mendoza, J. L., Taxonera, C., Fernandez-Arquero, M., Diaz-Rubio, M., de la Concha, E. G., and Urcelay, E. (2009) Role of ATG16L1 Thr300Ala Polymorphism in Inflammatory Bowel Disease: A Study in the Spanish Population and a Meta-analysis. *Inflamm Bowel Dis* **15**, 1697-1704

22. Zhang, H. F., Qiu, L. X., Chen, Y., Zhu, W. L., Mao, C., Zhu, L. G., Zheng, M. H., Wang, Y., Lei, L., and Shi, J. (2009) ATG16L1 T300A polymorphism and Crohn's disease susceptibility: evidence from 13,022 cases and 17,532 controls. *Hum Genet* **125**, 627-631

23. Plantinga, T. S., Joosten, L. A., van der Meer, J. W., and Netea, M. G. (2012) Modulation of inflammation by autophagy: consequences for Crohn's disease. *Curr Opin Pharmacol*

24. Plantinga, T. S., Crisan, T. O., Oosting, M., van de Veerdonk, F. L., de Jong, D. J., Philpott, D. J., van der Meer, J. W., Girardin, S. E., Joosten, L. A., and Netea, M. G. (2011) Crohn's disease-associated ATG16L1 polymorphism modulates pro-inflammatory cytokine responses selectively upon activation of NOD2. *Gut* **60**, 1229-1235

25. Crisan, T. O., Plantinga, T. S., van de Veerdonk, F. L., Farcas, M. F., Stoffels, M., Kullberg, B. J., van der Meer, J. W.,

Joosten, L. A., and Netea, M. G. (2011) Inflammasome-independent modulation of cytokine response by autophagy in human cells. *PLoS One* **6**, e18666

26. Moore, M. W., Cruz, A. R., LaVake, C. J., Marzo, A. L., Eggers, C. H., Salazar, J. C., and Radolf, J. D. (2007) Phagocytosis of Borrelia burgdorferi and Treponema pallidum potentiates innate immune activation and induces gamma interferon production. *Infect Immun* **75**, 2046-2062

27. Scherz-Shouval, R., Shvets, E., Fass, E., Shorer, H., Gil, L., and Elazar, Z. (2007) Reactive oxygen species are essential for autophagy and specifically regulate the activity of Atg4. *EMBO J* **26**, 1749-1760

28. Huang, J., and Brumell, J. H. (2009) NADPH oxidases contribute to autophagy regulation. *Autophagy* **5**, 887-889

29. Nakagawa, I., Amano, A., Mizushima, N., Yamamoto, A., Yamaguchi, H., Kamimoto, T., Nara, A., Funao, J., Nakata, M., Tsuda, K., Hamada, S., and Yoshimori, T. (2004) Autophagy defends cells against invading group A Streptococcus. *Science* **306**, 1037-1040

30. Gutierrez, M. G., Master, S. S., Singh, S. B., Taylor, G. A., Colombo, M. I., and Deretic, V. (2004) Autophagy is a defense mechanism inhibiting BCG and Mycobacterium tuberculosis survival in infected macrophages. *Cell* **119**, 753-766

31. Kleinnijenhuis, J., Oosting, M., Plantinga, T. S., van der Meer, J. W., Joosten, L. A., Crevel, R. V., and Netea, M. G. (2011) Autophagy modulates the Mycobacterium tuberculosis-induced cytokine response. *Immunology* **134**, 341-348

32. Saitoh, T., Fujita, N., Jang, M. H., Uematsu, S., Yang, B. G., Satoh, T., Omori, H., Noda, T., Yamamoto, N., Komatsu, M., Tanaka, K., Kawai, T., Tsujimura, T., Takeuchi, O., Yoshimori, T., and Akira, S. (2008) Loss of the autophagy protein Atg16L1 enhances endotoxin-induced IL-1beta production. *Nature* **456**, 264-268

33. Franchi, L., Eigenbrod, T., Munoz-Planillo, R., and Nunez, G. (2009) The inflammasome: a caspase-1-activation platform that regulates immune responses and disease pathogenesis. *Nat Immunol* **10**, 241-247

34. Jha, S., and Ting, J. P. (2009) Inflammasome-associated nucleotide-binding domain, leucine-rich repeat proteins and inflammatory diseases. *J Immunol* **183**, 7623-7629

35. Vandanmagsar, B., Youm, Y. H., Ravussin, A., Galgani, J. E., Stadler, K., Mynatt, R. L., Ravussin, E., Stephens, J. M., and Dixit, V. D. (2011) The NLRP3 inflammasome instigates obesity-induced inflammation and insulin resistance. *Nat Med* **17**, 179-188

36. Shi, C. S., Shenderov, K., Huang, N. N., Kabat, J., Abu-Asab, M., Fitzgerald, K. A., Sher, A., and Kehrl, J. H. (2012) Activation of autophagy by inflammatory signals limits IL-1beta production by targeting ubiquitinated inflammasomes for destruction. *Nat Immunol* **13**, 255-263

37. Netea, M. G., Nold-Petry, C. A., Nold, M. F., Joosten, L. A., Opitz, B., van der Meer, J. H., van de Veerdonk, F. L., Ferwerda, G., Heinhuis, B., Devesa, I., Funk, C. J., Mason, R. J., Kullberg, B. J., Rubartelli, A., van der Meer, J. W., and Dinarello, C. A. (2009)

Differential requirement for the activation of the inflammasome for processing and release of IL-1beta in monocytes and macrophages. *Blood* **113**, 2324-2335

38. Tosato, G., and Jones, K. D. (1990) Interleukin-1 induces interleukin-6 production in peripheral blood monocytes. *Blood* **75**, 1305-1310

39. Campbell, J., Ciesielski, C. J., Hunt, A. E., Horwood, N. J., Beech, J. T., Hayes, L. A., Denys, A., Feldmann, M., Brennan, F. M., and Foxwell, B. M. (2004) A novel mechanism for TNF-alpha regulation by p38 MAPK: involvement of NF-kappa B with implications for therapy in rheumatoid arthritis. *J Immunol* **173**, 6928-6937

40. Hsu, H. Y., and Wen, M. H. (2002) Lipopolysaccharide-mediated reactive oxygen species and signal transduction in the regulation of interleukin-1 gene expression. *J Biol Chem* **277**, 22131-22139

41. Segovia, J., Sabbah, A., Mgbemena, V., Tsai, S. Y., Chang, T. H., Berton, M. T., Morris, I. R., Allen, I. C., Ting, J. P., and Bose, S. (2012) TLR2/MyD88/NF-kappaB pathway, reactive oxygen species, potassium efflux activates NLRP3/ASC inflammasome during respiratory syncytial virus infection. *PLoS One* **7**, e29695

42. Delgado, M. A., Elmaoued, R. A., Davis, A. S., Kyei, G., and Deretic, V. (2008) Toll-like receptors control autophagy. *EMBO J* **27**, 1110-1121

43. Lee, J., Kim, H. R., Quinley, C., Kim, J., Gonzalez-Navajas, J., Xavier, R., and Raz, E. (2012) Autophagy suppresses interleukin-1beta (IL-1beta) signaling by activation of p62 degradation via lysosomal and proteasomal pathways. *J Biol Chem* **287**, 4033-4040

44. Sachs, G., Scott, D. R., Weeks, D. L., Rektorscheck, M., and Melchers, K. (2001) Regulation of Urease for Acid Habitation. in *Helicobacter pylori: Physiology and Genetics* (Mobley, H. L. T., Mendz, G. L., and Hazell, S. L. eds.), Washington (DC). pp

45. Huang, L. Y., Reis e Sousa, C., Itoh, Y., Inman, J., and Scott, D. E. (2001) IL-12 induction by a TH1-inducing adjuvant in vivo: dendritic cell subsets and regulation by IL-10. *J Immunol* **167**, 1423-1430

46. Geboes, L., Dumoutier, L., Kelchtermans, H., Schurgers, E., Mitera, T., Renauld, J. C., and Matthys, P. (2009) Proinflammatory Role of the Th17 Cytokine Interleukin-22 in Collagen-Induced Arthritis in C57BL/6 Mice. *Arthritis Rheum-Us* **60**, 390-395

47. Ma, H. L., Liang, S., Li, J., Napierata, L., Brown, T., Benoit, S., Senices, M., Gill, D., Dunussi-Joannopoulos, K., Collins, M., Nickerson-Nutter, C., Fouser, L. A., and Young, D. A. (2008) IL-22 is required for Th17 cell-mediated pathology in a mouse model of psoriasis-like skin inflammation. *J Clin Invest* **118**, 597-607

Chapter 4

Autophagy suppresses host adaptive immune responses towards *Borrelia burgdorferi*

J Leukoc Biol. 2016 Apr 21. pii: jlb.4A0715-331R

Kathrin Buffen, Marije Oosting, Yang Li, Thirumala-Devi Kanneganti, Mihai G. Netea, and Leo A.B. Joosten

Abstract

We have previously demonstrated that inhibition of autophagy increased the *Borrelia burgdorferi* induced innate cytokine production *in vitro*, but little is known regarding the effect of autophagy on *in vivo* models of *Borrelia* infection. Here we showed that ATG7 deficient mice that were intraarticular injected with *Borrelia* spirochetes displayed increased joint swelling, cell influx and enhanced IL-1β and IL-6 production by inflamed synovial tissue. Since both IL-1β and IL-6 are linked to the development of adaptive immune responses, we examine the function of autophagy on *Borrelia* induced adaptive immunity. Human peripheral blood mononuclear cells treated with autophagy inhibitors showed an increase in IL-17, IL-22 and IFN-γ production in response to exposure of *B. burgdorferi.* Increased IL-17 production was dependent on IL-1β release, but interestingly not on IL-23 production. In addition, cytokine QTLs in ATG9B modulate the *Borrelia*-induced IL-17 production. Since high levels of IL-17 have been found in patients with confirmed severe chronic borreliosis, we propose that the modulation of autophagy may be a potential target for anti-inflammatory therapy in patients with persistent Lyme Disease.

Introduction

Borrelia burgdorferi sensu lato, the causative agent of Lyme disease, stimulates a complex series of inflammatory events to eliminate the spirochete following infection [1]. The first most common sign of infection manifests as erythema migrans (EM) skin lesions, frequently accompanied by flu-like symptoms [2]. If treated correctly, the prognosis of these patients is excellent; however, if untreated, hematogenous dissemination of spirochetes may give rise to a wide range of clinical manifestations involving the central nervous system (mainly caused by *B. garinii*), the skin (*B. afzelii*) or the joints (*B. burgdorferi*) [3, 4].

Cytokines play a very important role in the pathogenesis of Lyme disease by regulating the immune responses against *Borrelia*. Several *in vitro* studies showed the important role of secreted IL-1β in response to *Borrelia* [5, 6]. High amounts of this cytokine were found near the location of erythema migrans lesions after tick bites [7]. IL-1 has a broad range of functions in mediating inflammation in protective immunity to infectious diseases, but it is also responsible for hyperinflammation in diseases associated with a dysregulated immune responses. IL-1β induces in synergy with IL-23 the production of IL-17 and related cytokines from Th17 cells [8], which have been associated to increased joint damage in rheumatoid arthritis or psoriasis patients [9, 10]. Next to that, IL-17 has been associated to the chronic stage of murine Lyme disease and inhibition of IL-17 by antibodies strongly reduced the development of Lyme arthritis shown by reduced joint swelling [11].

Previous studies have shown that autophagy, a highly conserved homeostatic mechanism that orchestrates the degradation of damaged cytosolic proteins, can modulate the IL-1β response to pathogens including *Borrelia* spirochetes [12]. Furthermore, inhibition of autophagy promotes the secretion of IL-1β which leads to elevated IL-17 levels after LPS stimulation [13].

In this report we investigated the role of autophagy in a knock-out mouse model of Lyme arthritis by injecting *B. burgdorferi* into the knee joints of the animals. ATG7 knock-out mice showed an increase in joint swelling, elevated cell influx into the joint cavity as well as increased cytokine levels. In addition, we investigated the role of autophagy on Borrelia-induced adaptive cytokines. The inhibition of autophagy by wortmannin increased the production of IL-17, IL-22 and IFN-γ in response to *Borrelia* bacteria. The increase of IL-17 was a specific response to elevated IL-1β levels and independent of elevated IL-23

levels. These findings underline the important role of autophagy in the pathogenesis of Lyme disease, and suggest that modulation of autophagy could be a novel therapeutic strategy in Lyme disease.

Methods

B. burgdorferi cultures. *B. burgdorferi,* ATCC strain 35210, was cultured at 33°C in Barbour-Stoenner-Kelley (BSK)-H medium (Sigma-Aldrich) supplemented with 6% rabbit serum. Spirochetes were grown to late-logarithmic phase and examined for motility by dark-field microscopy. Organisms were quantitated by fluorescence microscopy after mixing 10 µL aliquots of culture material with 10 µL of an acridine orange solution and counted using a Petroff-Hauser counting chamber. Bacteria were harvested by centrifugation of the culture at 7000 x g for 15 min., washed twice with sterile PBS (pH 7.4), and diluted in the specified medium to required concentrations of 1×10^6 spirochetes per ml.

Animals. All mice were maintained at St. Jude Children's Research Hospital (kindly provided by Douglas R. Green, St. Jude Children's Research Hospital, Memphis, TN, USA) and were described before [14]. Mice were housed in a specific pathogen-free (SPF) facility, and experiments were conducted under protocols approved by the St. Jude Children's Research Hospital's Committee on the Use and Care of Animals.

Isolation of human peripheral blood mononuclear cells and in vitro cytokine production. Venous blood was drawn from the cubital vein of healthy volunteers into 10 ml EDTA tubes (Monoject, Covidien, Mansfield, Massachusetts, USA). The mononuclear cell fraction was obtained by density centrifugation of blood diluted 1:1 in pyrogen-free saline over Ficoll-Paque (Pharmacia Biotech, Pittsburgh, Pennsylvania, USA). Cells were washed twice in saline and suspended in culture medium (RPMI; Invitrogen, Carlsbad, California, USA) supplemented with gentamicin 50 mg/ml, L-glutamine 2 mM and pyruvate 1 mM. Cells were counted in a Coulter counter (Coulter Electronics, Brea, California, USA) and the number was adjusted to $5x10^6$ cells/ ml. A total of $5x10^5$ mononuclear cells in a 100 µl volume was added to round-bottom 96-well plates (Greiner, Monroe, North Carolina, USA) and incubated with either 100 µl of culture medium (negative control), or *B. burgdorferi* (10^6 spirochetes per ml). In some experiments, PBMCs were pre-incubated with culture medium or the autophagy inhibitor wortmannin (100nM) for 60 minutes. At indicated time

points, supernatants were collected and stored at −20°C until being assayed.

CD4, CD8, CD56 depletion. To deplete cells from isolated PBMCs, cell subpopulations were labeled using magnetic beads coated with anti-CD4, anti-CD8 or anti-CD56 (MACS; Miltenyi Biotec, Germany). Subsequently, cells were depleted over a depletion column according to the protocol supplied by the manufacturer. As control for the isolation procedure, non-depleted PBMCs were also washed over the columns without coated beads.

Cytokine measurements. Concentrations of human IL-1α, IL-1β, IL-1ra, IL-23, TNFα, IL-17A, IL17F, IL-22, or IFNγ were determined in duplicates using specific commercial ELISA kits (Sanquin, Amsterdam, or R&D Systems, Minneapolis), in accordance with the manufacturers' instructions.

Real-time PCR. RNA from PBMCs was isolated using TRIzol reagent (Invitrogen) following the manufacturer's instructions. Isolated RNA was reversed transcribed into complementary DNA using iScript cDNA synthesis kit (Bio-Rad Laboratories BV, Veenendaal, the Netherlands). Quantitative real-time PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems) using a 7300 Real-time PCR system (Applied Biosystems). In each PCR reaction a melting curve analysis was included to control for aspecific PCR amplification. Primers used for the experiments (final concentration 10μM) are shown below. Real-time qPCR data were corrected for expression of the housekeeping gene human B2M. Human IL-1β; Forward sequence 5'-3': GCC-CTA-AAC-AGA-TGA-AGT-GCT-C, Reversed sequence 5'-3': GAA-CCA-GCA-TCT-TCC-TCA-G, Human IL-23; Forward sequence 5'-3': CAG-CTT-CAT-GCC-TCC-CTA-CT, Reversed sequence 5'-3': GAC-TGA-GGC-TTG-GAA-TCT-GC, Human TNF-α; Forward sequence 5'-3': TGG-CCC-AGG-CAG-TCA-GA, Reversed sequence 5'-3': GGT-TTG-CTA-CAA-CAT-GGG-CTA-CA, Human B2M; Forward sequence 5'-3': ATG-AGT-ATG-CCT-GCC-GTG-TG, Reversed sequence 5'-3': CCA-AAT-GCG-GCA-TCT-TCA-AAC, human IL-17A; Forward sequence 5'-3': CAA-TCC-CAA-AAG-GTC-CTC-AG, Reversed sequence 5'-3': CAC-TTT-GCC-TCC-CAG-ATC-A, human IL-17F; Forward sequence 5'-3': GGC-ATC-ATC-AAT-GAA-AAC-CA, Reversed sequence 5'-3': CTG-TAC-AAC-TTC-CGA-GGG-GTA, human IL-22; Forward sequence 5'-3': CAG-CAG-CCC-TAT-ATC-ACC-AA, Reversed sequence 5'-3': GGA-ACA-GTT-TCT-CCC-CAA-TG, human IFN-γ; Forward sequence 5'-3': CGA-GAT-GAC-TTC-GAA-AAG-CTG, Reversed sequence 5'-3': CAG-TTC-AGC-CAT-CAC-TTG-GA. Cycling conditions were 2 min at 50°C and 10 min at 95°C followed by 40 cycli of 95°C for 15 sec and 1 min at 60°C.

Intracellular IL-17, IL-22 and IFN-γ flow cytometry. Following 7 days stimulation, PBMCs were stimulated for 4-6 hours with PMA (50ng/ml) (Sigma-Aldrich), ionomycin (1µg/ml) (Sigma-Aldrich) and GolgiPlug (BD Biosciences) according to the protocols supplied by the manufacturers. Cells were stained extracellular using APC-CD4, FITC-CD4, PE-CD56, PECy7-CD56, ECD-CD8, PE-Cy7-CD45 or APC-CD45 antibodies (BD Biosciences or Beckman Coulter). Subsequently, cells were fixed and permeabilized with Cytofix/Cytoperm solution (eBioscience) according to the protocols supplied by the manufacturer. Following permeabilization, cells were stained intracellularly with FITC-conjugated anti-IL-17, FITC-conjugated anti-IFN-γ or PE-conjugated anti-IL-22 (BD Pharmingen or R&D) according to the protocols supplied by the manufacturers. The cells were measured on a FC500 flow cytometer (Beckman Coulter) and the data were analyzed using Kaluza 1.3.

Cytokine QTL mapping. Genotype and cytokine data could be generated for 391 healthy individuals. Gender information were coded either 0 for females or 1 for males. The actual age and coded gender information were included as co-variables in the linear regression model for cQTL mapping. Raw cytokine levels were log-transformed then correlated with genotype data. A nominal p value ≤ 0,05 indicates a suggestive cytokine QTL.

Patella washouts and cytokine measurements. Protein levels of murine IL-1β, IL-6 or KC were measured in patellae washouts. 4h after injection of 10^7 *Borrelia* spirochetes, patellae were isolated from inflamed knee joints and put in 0,05% Triton-X. After two freeze/thaw cycles, supernatant was harvested and centrifugated for 5 minutes at 10000rpm. Cytokines were determined by Luminex technology, kits were obtained from Bio-Rad (USA).

Histological analysis. Whole knee joints were removed and fixed in 4% formaldehyde for 7 days before decalcification in 5% formic acid and processing for paraffin embedding. Tissue sections (7µm) were stained with Haematoxylin/Eosin. Histopathological changes in the knee joints were scored in the patellae/femur region.

Ethics statement. All human experiments were conducted according to the principles expressed in the Declaration of Helsinki. Before taking blood, informed written consent of each human subject was provided. The study was approved by the review board of Radboud University Nijmegen Medical Centre.

Statistical Analysis. Data are expressed as mean ± SEM unless otherwise indicated.

Differences between experimental groups were tested using the two-sided Mann-Whitney *U* test performed on GraphPad Prism 4.0 software (GraphPad). *P* values of ≤0.05 were considered significant.

Results

***Borrelia*-induced joint inflammation is controlled by autophagy.** As described before, autophagy modulates *B. burgdorferi*-induced cytokine responses *in vitro*. To assess its role *in vivo*, we induced murine Lyme arthritis by injecting live spirochetes into knee joints of WT or ATG7-deficient mice. Lyme arthritis, detected as joint swelling of the injected knee, could be seen in WT mice, but was significantly increased in ATG7 knock-out mice (Figure 1A). In addition to joint swelling, cytokine levels were measured in patella washouts. Significant differences in IL-1β, IL-6 and KC production could be detected when WT patellae were compared with ATG7-deficient patellae (Figure 1B-D). In addition, cell influx into the joint cavity at 4h was assessed. In WT mice, only few cells infiltrated into the joint cavity in contrast to ATG7-ko mice, in which more cells (mainly neutrophils) could be found (Figure 1E+F).

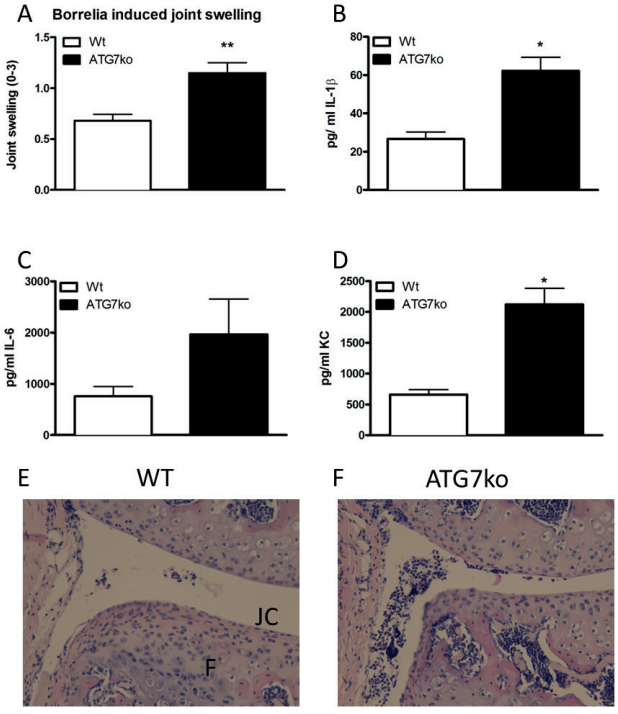


Figure 1. *Borrelia*-induced cytokine production and cell influx is modulated by autophagy. (A) Macroscopic score of the knees in either wild-type (WT) (white bars), or ATG7 knockout mice (black bars) 24 hours after intra-articular injection of 1x10^7 live *Borrelia* species in 10 µl PBS. Data are mean ± S.E. from five animals in each group; **P <0.01; Mann-Whitney U test, two-tailed. (B-D) 4 hours after i.a. injection of 1x 10^7 live *B. burgdorferi*, patellae were cultured for 1 hour and IL-1β, IL-6 and KC protein levels were measured using Luminex. Data are mean ± S.E.; 5 animals in each group, *, p < 0.05; **, p < 0.01; Mann-Whitney U-test, two-sided. (E) Murine Lyme arthritis in WT, or ATG7 knockout mice. Histology (H&E staining) after i.a. injection of *B. burgdorferi* in knee joints. Left panel, WT; right panel, ATG7 knockout mice. JC, joint cavity; F, femur.

Time dependent cytokine production of PBMCs stimulated with *B. burgdorferi*. To investigate the effect of autophagy on *B. burgdorferi* induced adaptive cytokines, we first measured the inflammatory response to *B. burgdorferi* itself, analyzing the levels of secreted cytokines by PBMCs stimulated with *B. burgdorferi* in a time-dependent manner. After exposure to *B. burgdorferi* spirochetes, we observed an early increase of IL-1β, IL-23, and TNF-α peaking after 24 hours (Figure 2A-C), as well as an increase in adaptive cytokines such as IL-17A, IL-17F, IL-22 and IFN-γ (Figure 2D-G).

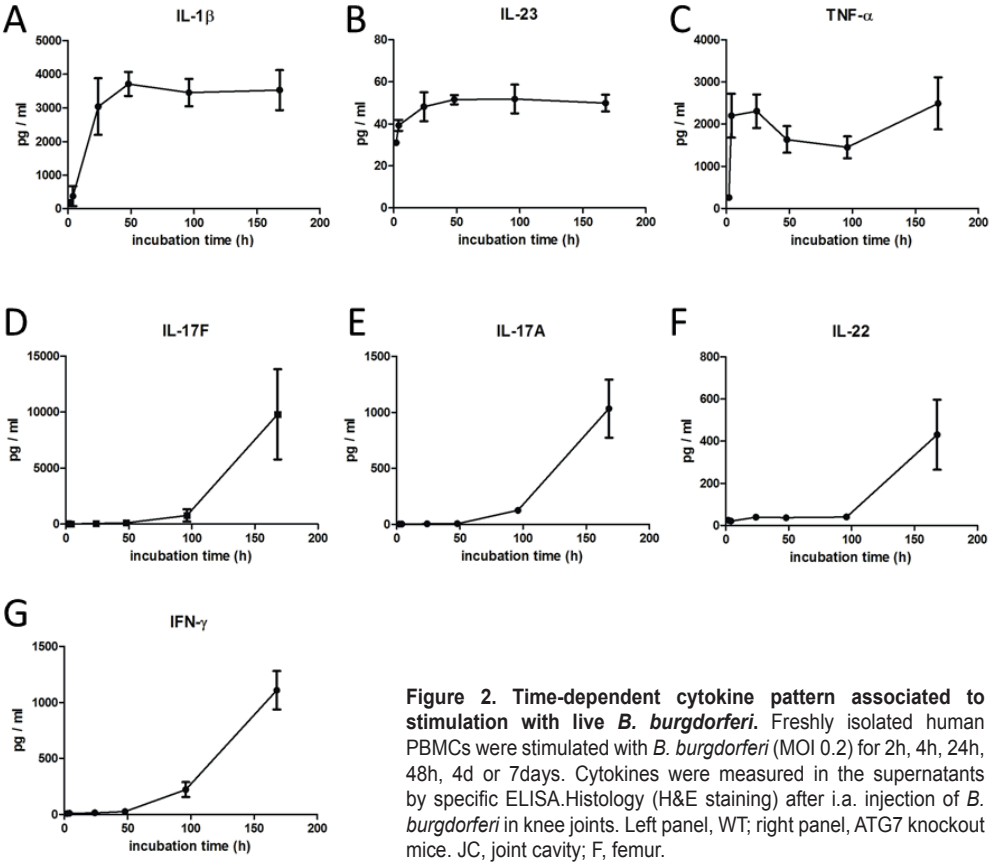


Figure 2. Time-dependent cytokine pattern associated to stimulation with live *B. burgdorferi*. Freshly isolated human PBMCs were stimulated with *B. burgdorferi* (MOI 0.2) for 2h, 4h, 24h, 48h, 4d or 7days. Cytokines were measured in the supernatants by specific ELISA.Histology (H&E staining) after i.a. injection of *B. burgdorferi* in knee joints. Left panel, WT; right panel, ATG7 knockout mice. JC, joint cavity; F, femur.

Inhibition of autophagy enhances innate and adaptive cytokines production. Previously, the modulating effect of autophagy on *B. burgdorferi*-induced IL-1β has been shown (Figure 3A) [12]. Since IL-1Ra is a known natural antagonist of IL-1β, we investigated the effect of autophagy on its production next to other innate cytokines

as IL-1α, TNF-α and IL-23. The inhibition of autophagy decreased the *B. burgdorferi*-induced IL-1Ra production (Figure 3B), shifting the IL-1β/IL-1ra ratio to a more prominent inflammatory response (Figure 3C). Interestingly, the production of IL-1α and TNF-α are not affected by wortmannin (Figure 3E, F). The increased amount of IL-23 in *B. burdorferi*-stimulated autophagy-incapable cells (Figure 3D) led us to investigate the effect of autophagy on adaptive cytokines, since IL-23 and IL-1β have been shown to be important in the production of IL-17[8, 15]. As shown in Figure 3G-J, the production of IL-17A, IL-17F, IL-22 as well as IFN-γ is significantly increased in autophagy-incapable cells stimulated with *B. burgdorferi*.

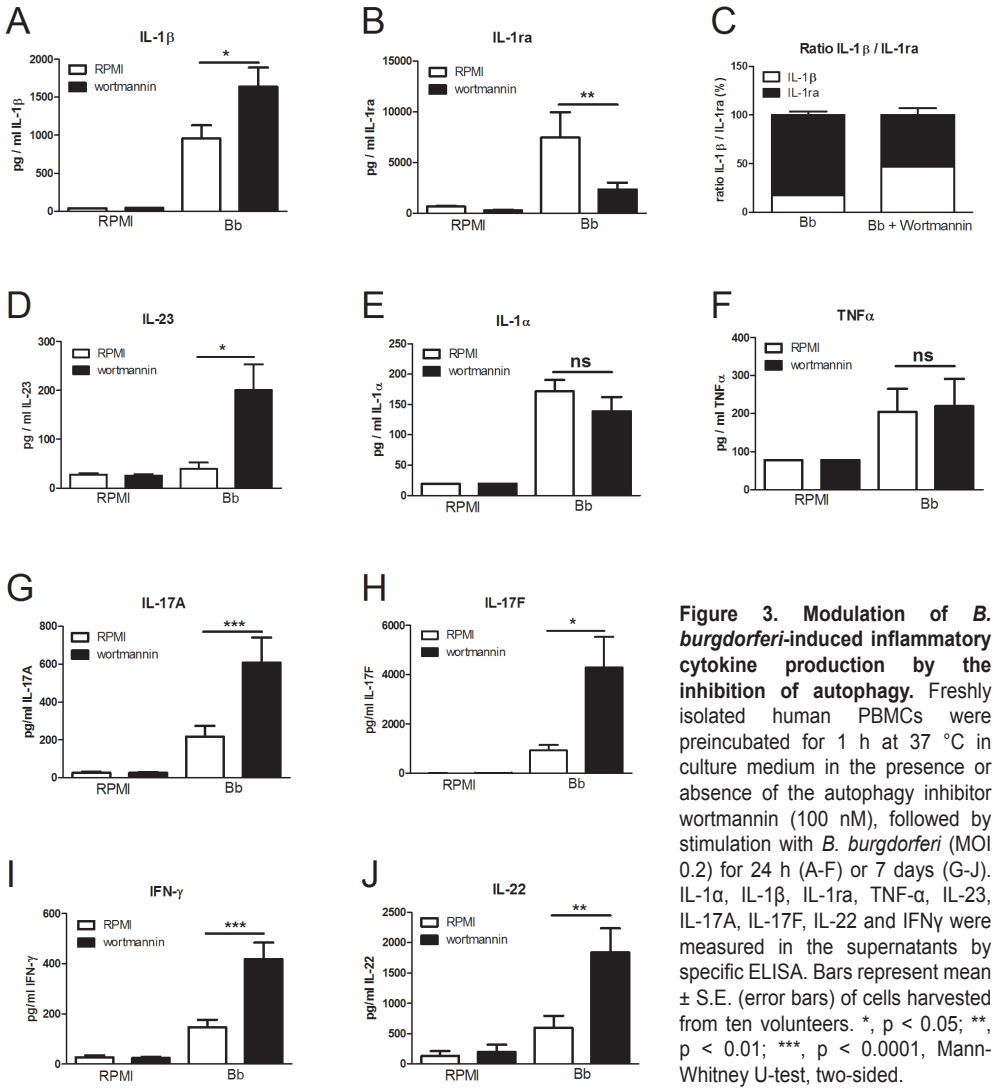


Figure 3. Modulation of *B. burgdorferi*-induced inflammatory cytokine production by the inhibition of autophagy. Freshly isolated human PBMCs were preincubated for 1 h at 37 °C in culture medium in the presence or absence of the autophagy inhibitor wortmannin (100 nM), followed by stimulation with *B. burgdorferi* (MOI 0.2) for 24 h (A-F) or 7 days (G-J). IL-1α, IL-1β, IL-1ra, TNF-α, IL-23, IL-17A, IL-17F, IL-22 and IFNγ were measured in the supernatants by specific ELISA. Bars represent mean ± S.E. (error bars) of cells harvested from ten volunteers. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.0001$, Mann-Whitney U-test, two-sided.

Inhibition of autophagy alters cytokine expression at the transcriptional level. To examine whether inhibition of autophagy during exposure to *Borrelia* results in altered mRNA levels, we measured transcription of several cytokines. After 24 hours, IL-1β and IL-23 mRNA levels were strongly increased in human *Borrelia* stimulated PBMCs when autophagy was inhibited (Figure 4A, B). As previously shown, the transcription of TNF-α was not altered (Figure 4C). The cytokine expression of IL-17A, IL-17F, IL-22 and IFN-γ was significantly increased after 4 days in autophagy-blocked cells (Figure 4D-G).

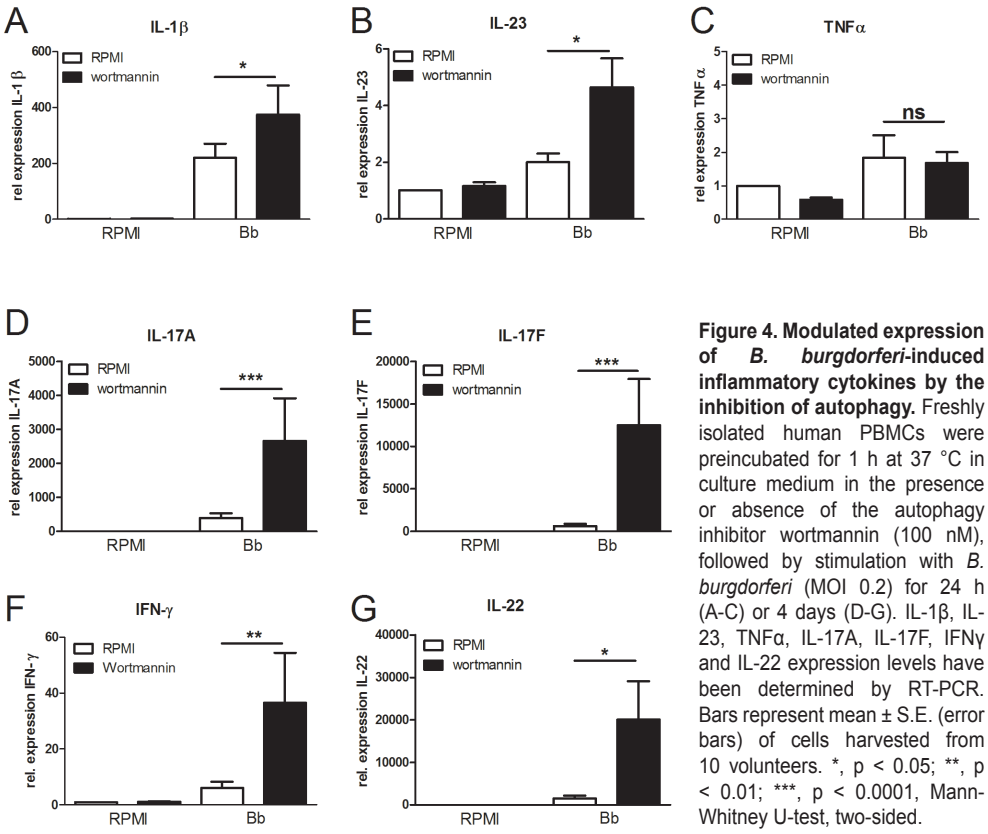


Figure 4. Modulated expression of *B. burgdorferi*-induced inflammatory cytokines by the inhibition of autophagy. Freshly isolated human PBMCs were preincubated for 1 h at 37 °C in culture medium in the presence or absence of the autophagy inhibitor wortmannin (100 nM), followed by stimulation with *B. burgdorferi* (MOI 0.2) for 24 h (A-C) or 4 days (D-G). IL-1β, IL-23, TNFα, IL-17A, IL-17F, IFNγ and IL-22 expression levels have been determined by RT-PCR. Bars represent mean ± S.E. (error bars) of cells harvested from 10 volunteers. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.0001$, Mann-Whitney U-test, two-sided.

The role of IL-1 and IL-23 in *B. burgdorferi*-induced IL-17, IL-22 and IFN-γ. In human PBMCs exposed to *B. burgdorferi*, IL-17 production is dependent on IL-1β and IL-23. To determine whether the increased cytokine response in autophagy-incapable cells was dependent on those cytokines, we blocked IL-1 and IL-23/IL-12 by specific antibodies before we stimulated the cells with *B. burgdorferi* in the presence or absence of wortmannin. As expected, IL-17 production was downregulated by inhibition of IL-1 and

IL-23 in *B. burgdorferi*-stimulated cells. Interestingly, the increase in the IL-17 production induced by autophagy inhibition was reduced in the presence of anti-IL-1 (Figure 5A), whereas the blockage of IL-23 had no effect (Figure 5B). The overall amount of IL-22 and IFN-γ was decreased in the presence of IL-1 antibodies (Figure 5C+E), but only IFN-γ production was downregulated by anti IL-23/IL-12 (Figure 5F); no effect on IL-22 production could be detected (Figure 5D).

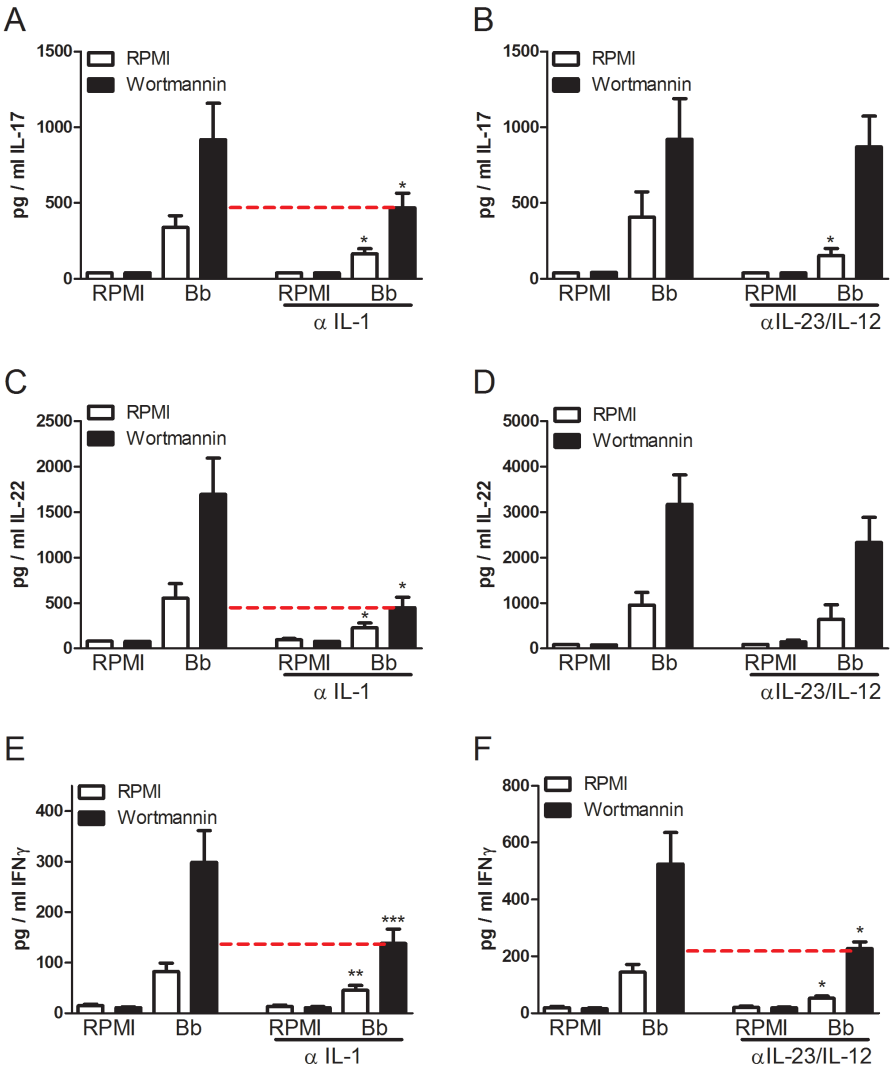
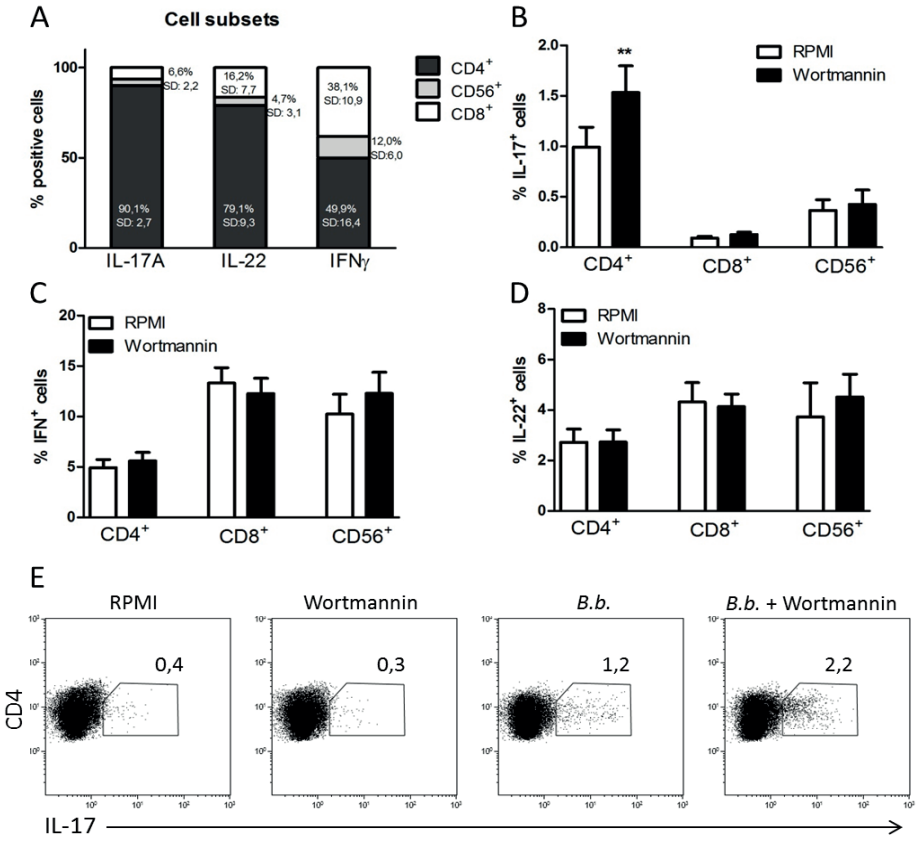


Figure 5. Role of IL-1β and IL-23 on autophagy-modulated *B. burgdorferi*-induced T cell responses. IL-17 (A, B), IL-22 (C, D) and IFN-γ (E, F) were measured in culture supernatants of PBMCS stimulated with *B. burgdorferi* for 7 days, in the presence or absence of the autophagy inhibitor wortmannin. IL-1 and IL-23/IL12 have been blocked by specific antibodies by 1 hour preincubation as indicated.

Increase of Th17 cells is responsible for boosted *B. burgdorferi*-induced IL-17A in autophagy incapable cells. In order to further investigate cellular sources of IL-17, IL-22 and IFN-γ in *B. burgdorferi*-stimulated cells, PBMCs were incubated for one week with the spirochete, before flow cytometry was performed for intracellular cytokines in CD4+, CD8+ or CD56+ cells. CD4+ cells are the main producers of *B. burgdorferi*-induced IL-17, whereas IFN-γ was produced by nearly equal amount of CD4+ and CD8+ cells. 12% of the overall IFN-γ production was due to CD56+ cells (Figure 6A). In addition, we wanted to assess whether the inhibition of autophagy increased a specific cell population that may be responsible for the increased cytokine response. A significant increase in CD4+IL-17+ cells (almost 50%) could be found in autophagy incapable cells stimulated with *B. burgdorferi*, explaining the increase in IL-17 production. Figure 6E demonstrates the increase of CD4+IL-17+ autophagy incapable PBMCs stimulated with *B. burgdorferi*. The percentages of CD8+IL17+ and CD56+IL-17+ cells did not differ between the stimulations (Figure 6B). Furthermore, no difference could be seen between the subpopulations being capable of producing IL-22 or IFN-γ (Figure 6C, D).



← page 76

Figure 6. Cellular source of autophagy modulated T cell responses induced by *B. burgdorferi*. (A) Assessment of surface markers CD4, CD56 and CD8 to elucidate the contribution of different cell types to the population of IL-17A+, IL-22+ and IFN-γ+ cells. (B-D) Comparison of the capacity of CD4+, CD8+ and CD56+ cells stimulated by *B. burgdorferi* in the presence or absence of wortmannin to produce the inflammatory cytokines IL-17, IFN-γ or IL-22. (E) Example of increased percentage of CD4+IL-17+ cells stimulated with *B. burgdorferi* in the presence of wortmannin compared to cells stimulated with *B. burgdorferi* in the absence of autophagy modulators.

CD4+ cells are the main producers of adaptive cytokines in response to *B. burgdorferi*. To proof our previous findings of the cellular sources of IL-17, IL-22 and IFN-γ, subpopulations known to be capable of producing these cytokines were depleted from the mixed cell population of PBMCs before exposure to *B. burgdorferi* in the presence or absence of wortmannin. For that purpose, cells expressing either CD4, CD8 or CD56 were targeted. After depletion, equal numbers of regular PBMC or depleted cell populations were preincubated with RPMI or wortmannin before stimulation with *B. burgdorferi*. Notably, CD4 depletion suppressed production of IL-17, IL-22 and IFN-γ in response to spirochetes down to background levels (Figure 7A-C).

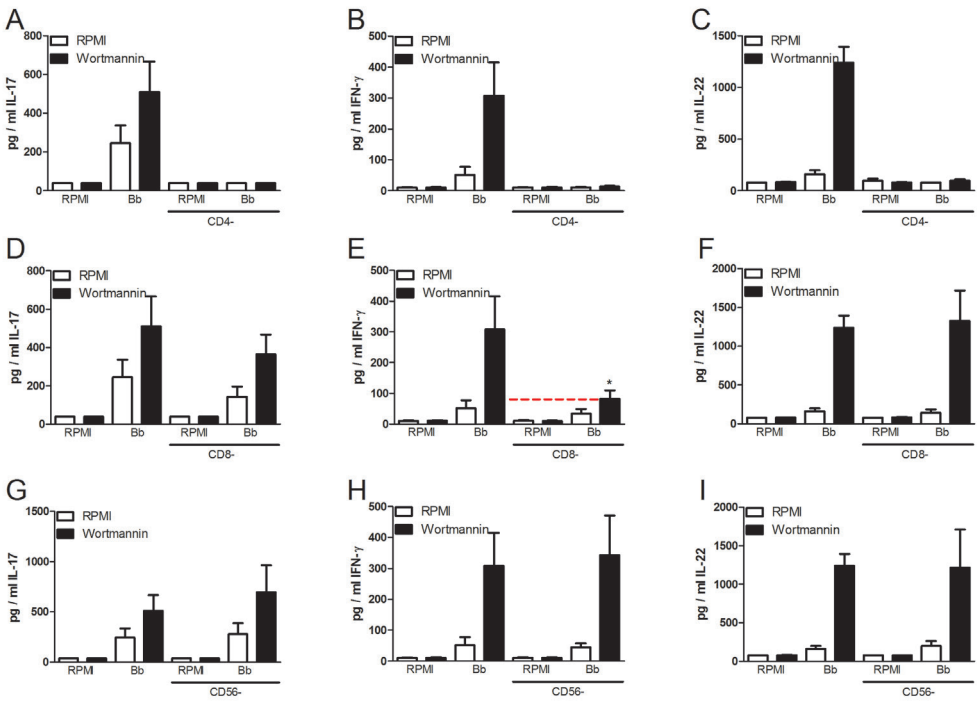


Figure 7. T cell dependent production of IL-17A, IL-22 and IFN-γ by PBMCs exposed to live *B. burgdorferi* in the presence or absence of an autophagy inhibitor. After isolation, PBMCs were both depleted for CD4, CD8 or CD56 and stimulated with *B. burgdorferi* in the presence or absence of wortmannin. Release of IL-17, IL-22 or IFN-γ was determined by ELISA after 7 days. Data are expressed as means ± S.E. (error bars) of cells harvested from 10 volunteers. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.0001$, Mann-Whitney U-test, two-sided.

CD8 depletion did only affect the amount of IFN-γ production, IL-17 and IL-22 were independent of the presence of CD8+ cells (Figure 7D-F). Depletion of CD56 had no effect on cytokine production at all (Figure7G-I).

cQTLs in ATG9B influence IL-17 production. Genetic factors are strongly influencing clinical disease outcome by having a huge regulatory impact on cytokine production [16]. Therefore, we assessed genetic variability in autophagy genes that influence *Borrelia*-induced cytokine production. Cytokine quantitative trait loci (cQTL) analysis was performed as described previously [17]. Two SNPs in ATG9B (rs10266701 and rs13307588) show suggestive cQTL ($p < 0.05$) for *Borrelia*-induced IL-17 (Figure 8A+B) underlining our previous findings of the importance of autophagy for *Borrelia*-induced cytokines.

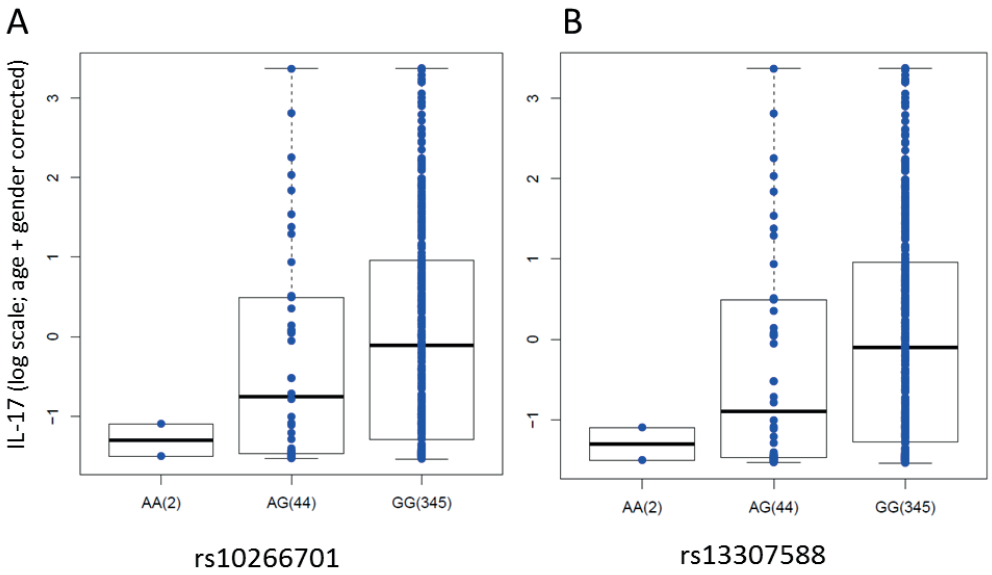


Figure 8. SNPs in ATG9B affect IL-17 levels induced by *B. burgdorferi*. Genetic variants in ATG9B (rs10266701 and rs13307588) modulates IL-17 levels induced by *B. burgdorferi*. Boxplots showing the association of genotypes at SNP (A) rs10266701(p=0,015) and (B) rs13307588 (p=0,028) with *B. burgdorferi* induced IL-17 levels. The number of individuals per genotype is shown in parenthesis below each boxplot.

Discussion

The connection between autophagy and innate defense mechanisms has been made in several studies describing a regulatory role of autophagy on inflammasome activation and production of cytokines upon stimulation with microbial ligands [12, 18-20]. Furthermore, it has been shown that autophagy modulates the production of T-cell derived cytokines as IL-17, IL-22 and IFN-γ [13, 21], all cytokines known to be produced also after stimulation with *B. burgdorferi* [15, 22]. Since IL-17 has been associated to increase joint damage in rheumatoid arthritis patients [9] and elevated IL-17 levels have been found in patients with confirmed neuroborreliosis [23], we examined the role of autophagy on the production of T cell-derived cytokines in response to *B. burgdorferi*.

In this study, we demonstrate that the secretion of adaptive inflammatory cytokines as IL-17, IL-22 and IFN-γ is highly elevated in autophagy-incapable PBMCs in response to *B. burgdorferi*. IL-1β and IL-23 are needed to induce Th17 responses [24-26], and therefore we investigated the role of autophagy in the modulation of their production. Elevated levels of IL-23 were produced by PBMCs in response to *B. burgdorferi* when autophagy was inhibited. Previous studies have shown that IL-23 promotes the development and expansion of Th17 cells. However, IL-23 alone cannot drive differentiation of those cells from naïve CD4+ T cell precursors. IL-1β signaling also has a critical role during the initial stages of Th17 cell differentiation; it enhances the metabolic fitness of rapidly dividing Th 17 cells during inflammation by the induction of phosphorylation of mTOR[27]. In our experiments, both cytokines are present to induce the development of IL-17. Apparently, the presence of IL-23 is important to promote the development of Th17 cells, but IL-1β is the key driver of the amount of IL-17 produced.

A previous study by Strle *et al*. [28] has shown elevated levels of IL-23 in patients with post-Lyme disease symptoms and elevated levels of IL-17 have been found in cerebrospinal fluid of neuroborreliose-patients [23]. It has been suggested that Th17 cells and their associated cytokines are involved in the pathogenesis of Lyme arthritis [11, 29, 30]. Defective autophagy would explain an increased Th17 response which might be associated to an increase in severity of disease symptoms.

Using flow cytometric analysis of PBMCs and depletion of CD4, CD8 or CD56 T-cell subsets, we determined that both cytokines IL-17 and IL-22 are primarily produced by

CD4+ T cells in response to *B. burgdorferi* stimulation. Next to its production by CD4+ cells, we were able to demonstrate that a significant number of CD8+ and CD56+ cells express IFN-γ in response to *B. burgdorferi,* which is in line with a previous report [31]. However, in contrast to this previous study, depletion of CD56+ cells did not alter *B. burgdorferi*-induced IFN-γ responses. Furthermore, the IFN+CD56+ cells did not expand upon stimulation with *B. burgdorferi*. These data suggest that NK cells do not have a major contribution to the IFN-γ response to *Borrelia* in this setting.

Next to our *in vitro* data, we injected *B. burgdorferi* into the knee joints of ATG7 deficient mice, showing increased joint swelling and more cell influx compared to WT mice. It is known that the genetic background of mice influences the susceptibility for several experimental disease models including the induction and maintenance of experimental murine Lyme disease. C57Bl/6 mice clear the infection very fast, which explains the mild joint swelling and hardly any cell influx due to the bacteria. Long-term effects on IL-17 production could not be measured since the infection is cleared soon after injection of the bacteria. To study the effect of autophagy on adaptive cytokines in mice, an ATG-knockout needs to be developed in a mouse strain that is more susceptible to *Borrelia* infection, such as the C3H/H3N mice.

In summary, we have demonstrated a regulatory link between autophagy and T-cell cytokine production in response to *B. burgdorferi* stimulation, which is dependent on IL-1 but not on IL-23 secretion. These findings further highlight autophagy as a potential target for anti-inflammatory therapies in Lyme disease.

References

1. Steere, A. C., Coburn, J., Glickstein, L. (2004) The emergence of Lyme disease. The Journal of clinical investigation 113, 1093-101.

2. Wormser, G. P., Dattwyler, R. J., Shapiro, E. D., Halperin, J. J., Steere, A. C., Klempner, M. S., Krause, P. J., Bakken, J. S., Strle, F., Stanek, G., Bockenstedt, L., Fish, D., Dumler, J. S., Nadelman, R. B. (2006) The clinical assessment, treatment, and prevention of lyme disease, human granulocytic anaplasmosis, and babesiosis: clinical practice guidelines by the Infectious Diseases Society of America. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 43, 1089-134.

3. Steere, A. C. (2001) Lyme disease. The New England journal of medicine 345, 115-25.

4. Balmelli, T. and Piffaretti, J. C. (1995) Association between different clinical manifestations of Lyme disease and different species of Borrelia burgdorferi sensu lato. Research in microbiology 146, 329-40.

5. Miller, L. C., Isa, S., Vannier, E., Georgilis, K., Steere, A. C., Dinarello, C. A. (1992) Live Borrelia burgdorferi preferentially activate interleukin-1 beta gene expression and protein synthesis over the interleukin-1 receptor antagonist. The Journal of clinical investigation 90, 906-12.

6. Miller, L. C., Lynch, E. A., Isa, S., Logan, J. W., Dinarello, C. A., Steere, A. C. (1993) Balance of synovial fluid IL-1 beta and IL-1 receptor antagonist and recovery from Lyme arthritis. Lancet 341, 146-8.

7. Jones, K. L., Muellegger, R. R., Means, T. K., Lee, M., Glickstein, L. J., Damle, N., Sikand, V. K., Luster, A. D., Steere, A. C. (2008) Higher mRNA levels of chemokines and cytokines associated with macrophage activation in erythema migrans skin lesions in patients from the United States than in patients from Austria with Lyme borreliosis. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 46, 85-92.

8. Oosting, M., ter Hofstede, H., van de Veerdonk, F. L., Sturm, P., Kullberg, B. J., van der Meer, J. W., Netea, M. G., Joosten, L. A. (2011) Role of interleukin-23 (IL-23) receptor signaling for IL-17 responses in human Lyme disease. Infection and immunity 79, 4681-7.

9. Geboes, L., Dumoutier, L., Kelchtermans, H., Schurgers, E., Mitera, T., Renauld, J. C., Matthys, P. (2009) Proinflammatory role of the Th17 cytokine interleukin-22 in collagen-induced arthritis in C57BL/6 mice. Arthritis and rheumatism 60, 390-5.

10. Ma, H. L., Liang, S., Li, J., Napierata, L., Brown, T., Benoit, S., Senices, M., Gill, D., Dunussi-Joannopoulos, K., Collins, M., Nickerson-Nutter, C., Fouser, L. A., Young, D. A. (2008) IL-22 is required for Th17 cell-mediated pathology in a mouse model of psoriasis-like skin inflammation. The Journal of clinical investigation 118, 597-607.

11. Burchill, M. A., Nardelli, D. T., England, D. M., DeCoster, D. J., Christopherson, J. A., Callister, S. M., Schell, R. F. (2003) Inhibition of interleukin-17 prevents the development of arthritis in vaccinated mice challenged with Borrelia burgdorferi. Infection and

immunity 71, 3437-42.

12. Buffen, K., Oosting, M., Mennens, S., Anand, P. K., Plantinga, T. S., Sturm, P., van de Veerdonk, F. L., van der Meer, J. W., Xavier, R. J., Kanneganti, T. D., Netea, M. G., Joosten, L. A. (2013) Autophagy modulates Borrelia burgdorferi-induced production of interleukin-1beta (IL-1beta). The Journal of biological chemistry 288, 8658-66.

13. Peral de Castro, C., Jones, S. A., Ni Cheallaigh, C., Hearnden, C. A., Williams, L., Winter, J., Lavelle, E. C., Mills, K. H., Harris, J. (2012) Autophagy regulates IL-23 secretion and innate T cell responses through effects on IL-1 secretion. Journal of immunology 189, 4144-53.

14. Smeekens, S. P., Malireddi, R. K., Plantinga, T. S., Buffen, K., Oosting, M., Joosten, L. A., Kullberg, B. J., Perfect, J. R., Scott, W. K., van de Veerdonk, F. L., Xavier, R. J., van de Vosse, E., Kanneganti, T. D., Johnson, M. D., Netea, M. G. (2014) Autophagy is redundant for the host defense against systemic Candida albicans infections. European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology 33, 711-22.

15. Oosting, M., van de Veerdonk, F. L., Kanneganti, T. D., Sturm, P., Verschueren, I., Berende, A., van der Meer, J. W., Kullberg, B. J., Netea, M. G., Joosten, L. A. (2011) Borrelia species induce inflammasome activation and IL-17 production through a caspase-1-dependent mechanism. European journal of immunology 41, 172-81.

16. Netea, M. G., van de Veerdonk, F. L., van der Meer, J. W. (2012) Primary immunodeficiencies of pattern recognition receptors. Journal of internal medicine 272, 517-27.

17. Li, Y., Oosting, M., Deelen, P., Ricano-Ponce, I., Smeekens, S., Jaeger, M., Matzaraki, V., Swertz M.A., Xavier, R.J., Franke, L., Wijmenga C., Joosten, L.A.B., Kumar, V., Netea M.G. (2016) The architecture and variability of cytokine responses against bacterial and fungal human pathogens. Nature Medicine in press.

18. Gutierrez, M. G., Master, S. S., Singh, S. B., Taylor, G. A., Colombo, M. I., Deretic, V. (2004) Autophagy is a defense mechanism inhibiting BCG and Mycobacterium tuberculosis survival in infected macrophages. Cell 119, 753-66.

19. Nakagawa, I., Amano, A., Mizushima, N., Yamamoto, A., Yamaguchi, H., Kamimoto, T., Nara, A., Funao, J., Nakata, M., Tsuda, K., Hamada, S., Yoshimori, T. (2004) Autophagy defends cells against invading group A Streptococcus. Science 306, 1037-40.

20. Kleinnijenhuis, J., Oosting, M., Plantinga, T. S., van der Meer, J. W., Joosten, L. A., Crevel, R. V., Netea, M. G. (2011) Autophagy modulates the Mycobacterium tuberculosis-induced cytokine response. Immunology 134, 341-8.

21. Said, A., Bock, S., Lajqi, T., Muller, G., Weindl, G. (2014) Chloroquine promotes IL-17 production by CD4+ T cells via p38-dependent IL-23 release by monocyte-derived Langerhans-like cells. Journal of immunology 193, 6135-43.

22. Bachmann, M., Horn, K., Rudloff, I., Goren, I., Holdener, M., Christen, U., Darsow,

N., Hunfeld, K. P., Koehl, U., Kind, P., Pfeilschifter, J., Kraiczy, P., Muhl, H. (2010) Early production of IL-22 but not IL-17 by peripheral blood mononuclear cells exposed to live Borrelia burgdorferi: the role of monocytes and interleukin-1. PLoS pathogens 6, e1001144.

23. Henningsson, A. J., Tjernberg, I., Malmvall, B. E., Forsberg, P., Ernerudh, J. (2011) Indications of Th1 and Th17 responses in cerebrospinal fluid from patients with Lyme neuroborreliosis: a large retrospective study. Journal of neuroinflammation 8, 36.

24. Sutton, C. E., Lalor, S. J., Sweeney, C. M., Brereton, C. F., Lavelle, E. C., Mills, K. H. (2009) Interleukin-1 and IL-23 induce innate IL-17 production from gammadelta T cells, amplifying Th17 responses and autoimmunity. Immunity 31, 331-41.

25. Zielinski, C. E., Mele, F., Aschenbrenner, D., Jarrossay, D., Ronchi, F., Gattorno, M., Monticelli, S., Lanzavecchia, A., Sallusto, F. (2012) Pathogen-induced human TH17 cells produce IFN-gamma or IL-10 and are regulated by IL-1beta. Nature 484, 514-8.

26. Chung, Y., Chang, S. H., Martinez, G. J., Yang, X. O., Nurieva, R., Kang, H. S., Ma, L., Watowich, S. S., Jetten, A. M., Tian, Q., Dong, C. (2009) Critical regulation of early Th17 cell differentiation by interleukin-1 signaling. Immunity 30, 576-87.

27. Gaffen, S. L., Jain, R., Garg, A. V., Cua, D. J. (2014) The IL-23-IL-17 immune axis: from mechanisms to therapeutic testing. Nature reviews. Immunology 14, 585-600.

28. Strle, K., Stupica, D., Drouin, E. E., Steere, A. C., Strle, F. (2014) Elevated levels of IL-23 in a subset of patients with post-lyme disease symptoms following erythema migrans. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 58, 372-80.

29. Kotloski, N. J., Nardelli, D. T., Peterson, S. H., Torrealba, J. R., Warner, T. F., Callister, S. M., Schell, R. F. (2008) Interleukin-23 is required for development of arthritis in mice vaccinated and challenged with Borrelia species. Clinical and vaccine immunology : CVI 15, 1199-207.

30. Codolo, G., Amedei, A., Steere, A. C., Papinutto, E., Cappon, A., Polenghi, A., Benagiano, M., Paccani, S. R., Sambri, V., Del Prete, G., Baldari, C. T., Zanotti, G., Montecucco, C., D'Elios, M. M., de Bernard, M. (2008) Borrelia burgdorferi NapA-driven Th17 cell inflammation in lyme arthritis. Arthritis and rheumatism 58, 3609-17.

31. Katchar, K., Drouin, E. E., Steere, A. C. (2013) Natural killer cells and natural killer T cells in Lyme arthritis. Arthritis research & therapy 15, R183.

Chapter 5

Transcriptomic and genomic analysis identify SNPs in autophagy genes that regulate the production of *Borrelia burgdorferi*-induced cytokines.

In preparation

Kathrin Buffen, Mariska Kerstholt, Rob ter Horst, Marije Oosting, Martin Jäger, Sanne P. Smeekens, Yang Li, Vinod Kumar, Cisca Wijmenga, Mihai G. Netea, Leo A.B. Joosten

Abstract

We have previously demonstrated that inhibition of autophagy increased the *Borrelia burgdorferi* induced cytokine production, but little is known about the molecular pathway behind this phenomenon. Here, by integrating transcriptional analysis and functional genomics, we identified the key players of autophagy-related genes which were modulated by stimulation with the *Borrelia* spirochete. To confirm the clinical relevance of these findings, we investigated the expression of autophagy-related genes in Lyme disease patients and compared the results to healthy controls. Several genes were differentially expressed highlighting the importance of the autophagy machinery during the course of Lyme disease. In addition, we identified around 200 cQTLs in autophagy-related genes that modulated the *Borrelia*-induced cytokine production. Since increased levels of cytokines as IL-17 have been found in patients with confirmed severe forms of borreliosis, we propose that the modulation of autophagy may be a potential target for anti-inflammatory therapy in patients with persistent Lyme Disease.

Introduction

Lyme disease, the most common vector-borne disease in the United States and Western Europe, is caused by spirochetes of the species *Borrelia burgdorferi sensu lato* [1]. The tick-born disease presents itself under a wide variety of clinical manifestations ranging from early localized symptoms as erythema migrans to more persistent ones involving a patient's skin (*B. afzelii*), joints (*B. burgdorferi*) or nervous system (*B. garinii*) [2,3].

The host's immune response against *B. burgdorferi* is regulated by several cytokines including IL-1β and IL-17 [4-6]. Both cytokines have a broad range of functions as mediating inflammation in protective immunity to infectious diseases, but are also responsible for hyperinflammation in diseases associated with a dysregulated immune response as increased joint damage in rheumatoid arthritis [7]. Recognition of spirochetes by different pattern recognition receptors lead next to the production of cytokines, also to the induction of autophagy, a process associated to the degradation of damaged organelles or protein [8]. When its signaling pathways are activated, autophagy starts with an ensemble of autophagy (ATG) factors inducing the formation of an isolation membrane. Thereafter, an autophagosome is formed, fuses with a lysosome to create an autolysosome, followed by the degradation of the cargo and release of degraded products back into the cytosol [9]. In addition to its known function, autophagy has been shown to regulate the production of inflammatory cytokines and immune responses in infection [10,11].

Assessment of clinical symptoms and immune characteristics of *Borrelia*-infected patients illustrates a large diversity in the size of EM lesions as well as in cytokine levels which have been associated to genetic variations in these patients leading to differences in clinical outcome [12,13]. Despite the importance of immune variation for the outcome of disease, only few studies have looked into genetic factors influencing cytokine production induced by *B. burgdorferi* [13]. In this report, we investigated the role of genetic variations in autophagy genes and their effect on *Borrelia*-induced cytokine responses. Different expression profiles of ATG genes could be found in Lyme disease patients compared to healthy controls. Furthermore, genetic variants of autophagy genes could be associated to different levels of *Borrelia*-induced cytokines. These findings underline the important role of autophagy in the pathogenesis of Lyme disease, suggesting that modulation of autophagy could be a novel target in the course of treatment of Lyme disease patients.

Material & Methods

B. burgdorferi cultures. *B. burgdorferi,* ATCC strain 35210, was cultured at 33°C in Barbour-Stoenner-Kelley (BSK)-H medium (Sigma-Aldrich) supplemented with 6% rabbit serum. Spirochetes were grown to late-logarithmic phase and examined for motility by dark-field microscopy. Organisms were quantitated by fluorescence microscopy after mixing 10 µL aliquots of culture material with 10 µL of an acridine orange solution and counted using a Petroff-Hauser counting chamber. Bacteria were harvested by centrifugation of the culture at 7000 x g for 15 min., washed twice with sterile PBS (pH 7.4), and diluted in the specified medium to required concentrations of 1×10^6 spirochetes per ml.

Isolation of human peripheral blood mononuclear cells and *in vitro* cytokine production. Venous blood was drawn from the cubital vein of healthy volunteers into 10 ml EDTA tubes (Monoject, Covidien, Mansfield, Massachusetts, USA). The mononuclear cell fraction was obtained by density centrifugation of blood diluted 1:1 in pyrogen-free saline over Ficoll-Paque (Pharmacia Biotech, Pittsburgh, Pennsylvania, USA). Cells were washed twice in saline and suspended in culture medium (RPMI; Invitrogen, Carlsbad, California, USA) supplemented with gentamicin 50 mg/ml, L-glutamine 2 mM and pyruvate 1 mM. Cells were counted in a Coulter counter (Coulter Electronics, Brea, California, USA) and the number was adjusted to $5x10^6$ cells/ ml. A total of $5x10^5$ mononuclear cells in a 100 µl volume was added to round-bottom 96-well plates (Greiner, Monroe, North Carolina, USA) and incubated with either 100 µl of culture medium (negative control), or *B. burgdorferi* (10^6 spirochetes per ml). At indicated time points, supernatants were collected and stored at −20°C until being assayed.

Cytokine measurements. Concentrations of human IL-1β, IL-6, TNFα, IL-17, IL-22, or IFNγ were determined in duplicates using specific commercial ELISA kits (Sanquin, Amsterdam, or R&D Systems, Minneapolis), in accordance with the manufacturers' instructions.

RNA sequencing. RNA sequencing data was obtained after stimulation of PBMCs with RPMI or *B. burgdorferi* for 4 hours. Sequencing reads were mapped to the human genome using STAR (version 2.3.0) [14]. The aligner was provided with a file containing junctions from Ensembl GRCh37.71. Htseq-count of the Python package HTSeq (version 0.5.4p3) was used (The HTSeq package, http://wwwhuber.embl.de/users/anders/HTSeq/

doc/overview. html) to quantify the read counts per gene based on annotation version GRCh37.71, using the default union-counting mode. Differentially expressed genes were identified by statistics analysis using DESeq2 package from bioconductor [15]. The statistically significant threshold (FDR P ≤ 0.05 and Fold Change ≥2) was applied.

Pathway enrichment analysis. Pathway enrichment analysis was performed using the Cytoscape app ClueGO [16]. Enrichment in a set of selected genes was checked for all path ways present in the Wiki-pathways and Reactome databases compared to a background set of all genes for which differential expression was calculated. Analysis was performed separately for the sets of up and down regulated genes. Pathways with a corrected (enrichment) P-value smaller than 0.05 are considered significant. P values were corrected using the Bonferroni step down algorithm built into the ClueGO app.

Lyme disease patients. RNAsequencing data of acute Lyme patients was obtained from a publicly available dataset ([17], accession number GSE63085) through the Gene Expression Omnibus (GEO) data repository. Expression of selected genes was compared between erythema migrans (EM) patients at different time points and healthy controls by Kruskal-Wallis one-way ANOVA with Dunn's post-hoc test-T.

Genotyping, quality control and imputation. DNA samples were genotyped as previously described [13] using the commercially available SNPchip, Illumina HumnaOmniExpressExome-8 v1.0.

Cytokine QTL mapping. Genotype and cytokine data could be generated for 391 healthy individuals. Gender information were coded either 0 for females or 1 for males. The actual age and coded gender information were included as co-variables in the linear regression model for cQTL mapping. Raw cytokine levels were log-transformed then correlated with genotype data. A nominal p value ≤ 0.05 indicates a suggestive cytokine QTL.

Ethics statement. All human experiments were conducted according to the principles expressed in the Declaration of Helsinki. Before taking blood, informed written consent of each human subject was provided. The study (nr. 42561.091.12) was approved by the review board of Radboud University Medical Centre, Nijmegen, The Netherlands.

Statistical Analysis. Data are expressed as mean ± SEM unless otherwise indicated.

Differences between experimental groups were tested using the two-sided Mann-Whitney *U* test performed on GraphPad Prism 4.0 software (GraphPad). *P* values of ≤0.05 were considered significant.

Results

***B. burgdorferi* stimulation induces the transcription of autophagy-related proteins.** To identify new targets for the treatment of Lyme disease, we investigated the transcriptional profile of *B. burgdorferi*-stimulated human PBMCs. Transcriptomic assessment of these PBMCs by RNA sequencing (Figure 1A) and pathway analysis (Figure 1B) revealed specific clusters of genes significantly induced by *B. burgdorferi*-stimulation with an intriguing signal found in genes associated to autophagy.

Therefore, we focused on autophagy related genes, to find the key players involved during the pathogenesis of *Borrelia*-infection. As shown in Figure 2A, 36 genes associated to autophagy were analyzed for up or downregulation in response to *Borrelia*-infection, whereof 12 genes were found to be significantly regulated (Figure 2B).

Autophagy genes are differentially expressed in Lyme disease patients. To further investigate the clinical impact of autophagy in Lyme disease, we looked into different expression profiles of autophagy-related genes in Lyme disease patients and compared the outcome to healthy controls. ATG3 (Figure 3A) and ATG7 (Figure 3B) were both significantly increased in patients compared to healthy controls. ATG9, ATG16L1 and mTOR expression (Figure 3C-F) were significantly downregulated in early Lyme disease patients (up to 3 weeks post *Borrelia* infection), but background levels were reached after 6 months post-infection. The results are in line with previous in vitro stimulation data, showing up- and downregulation of autophagy related genes due to *B. burgdorferi* stimulation.

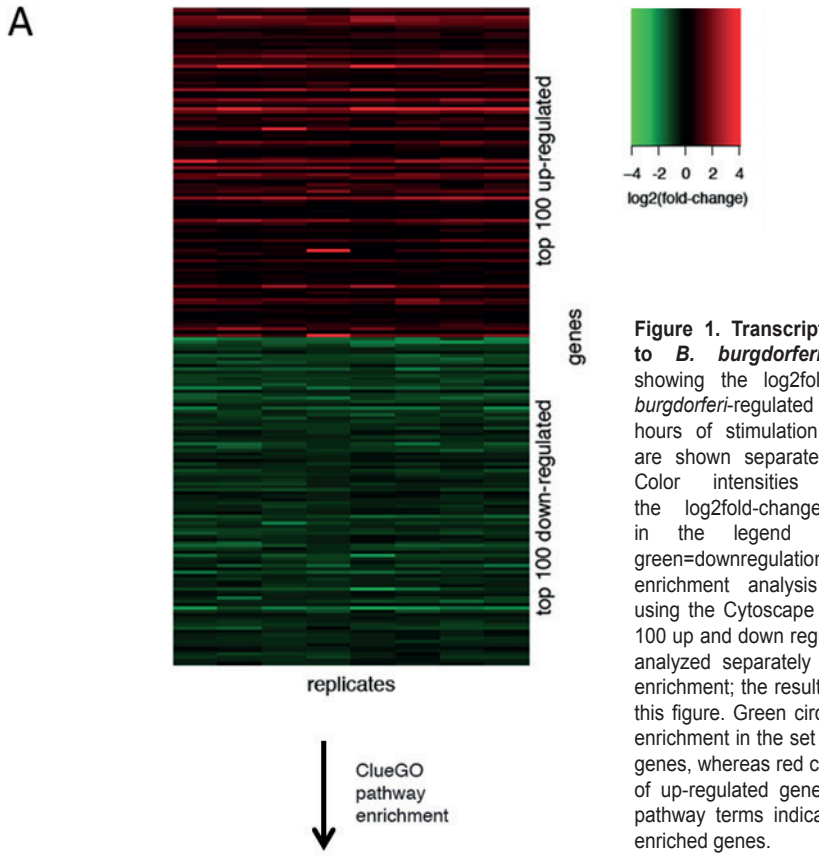
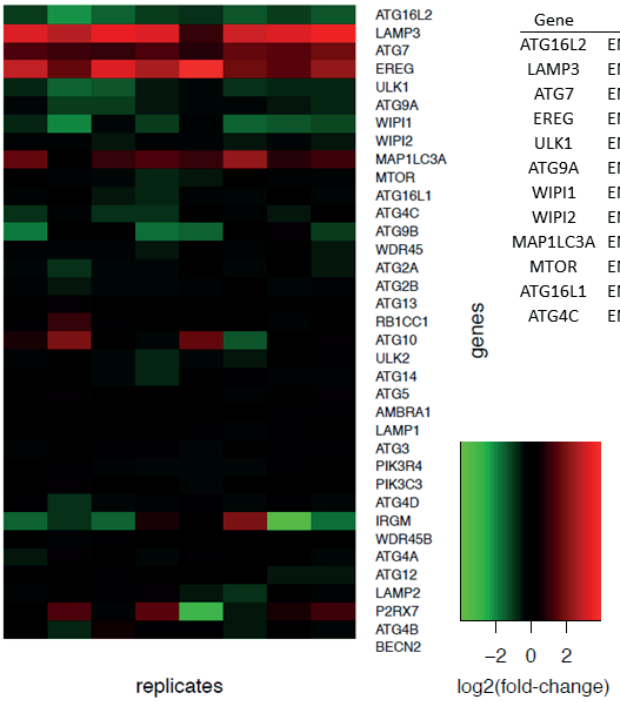


Figure 1. Transcriptional responses to *B. burgdorferi*. (A) Heatmap showing the log2fold-changes of *B. burgdorferi*-regulated genes after 4 hours of stimulation. Eight replicates are shown separately on the x-axis. Color intensities correspond to the log2fold-changes as indicated in the legend (red=upregulation, green=downregulation). (B) Pathway enrichment analysis was performed using the Cytoscape app ClueGO. Top 100 up and down regulated genes were analyzed separately for their pathway enrichment; the results are combined in this figure. Green circles correspond to enrichment in the set of down-regulated genes, whereas red circles indicate sets of up-regulated genes. Lines between pathway terms indicate similar sets of enriched genes.

A



B

Gene	ensemble ID	log2FoldChange	padj
ATG16L2	ENSG00000168010	-0,90	3,6E-15
LAMP3	ENSG00000078081	2,23	1,6E-14
ATG7	ENSG00000197548	1,02	1,8E-09
EREG	ENSG00000124882	1,95	1,4E-07
ULK1	ENSG00000177169	-0,55	1,0E-05
ATG9A	ENSG00000198925	-0,41	2,6E-05
WIPI1	ENSG00000070540	-0,71	3,4E-04
WIPI2	ENSG00000157954	-0,22	1,1E-03
MAP1LC3A	ENSG00000101460	0,91	1,1E-03
MTOR	ENSG00000198793	-0,19	3,3E-03
ATG16L1	ENSG00000085978	-0,19	1,4E-02
ATG4C	ENSG00000125703	-0,31	9,8E-02

Figure 2. Expression of autophagy genes after *Borrelia burgdorferi* recognition. (A) Heatmap showing fold-changes for a selected set of autophagy genes, with each of the replicates shown separately. Genes are sorted top to bottom with decreasing P-values. (B) Table showing autophagy genes significantly up and downregulated by *B. burgdorferi*.

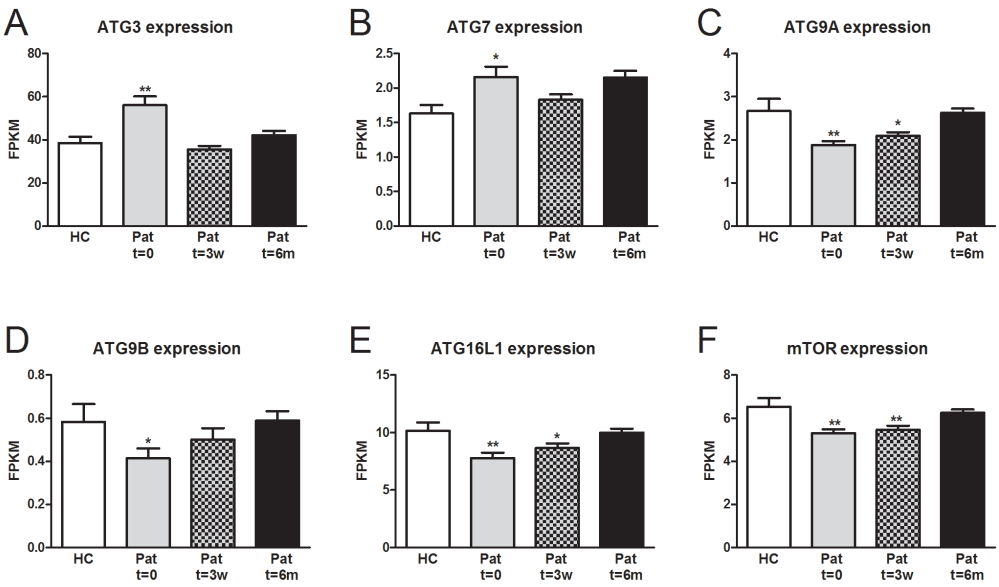
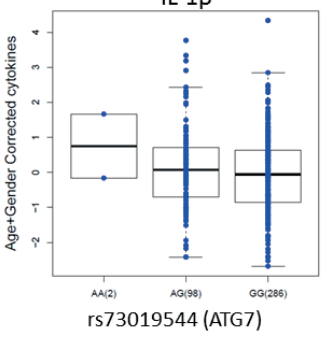


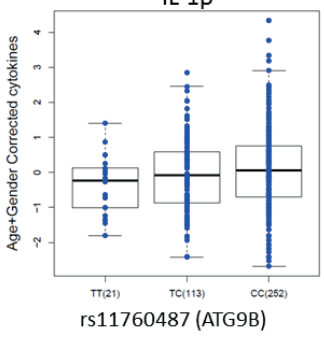
Figure 3. Autophagy gene expression profiles in Lyme patients compared to healthy controls. The expression of several autophagy genes – (A-F) ATG3, ATG7, ATG9A, ATG9B, ATG16L1 and mTOR - in PBMCs isolated from heathy controls and patients showing erythema migrans (time points: 0, 3 weeks, 6 months) were analyzed. Bars represent mean ± S.E.M. (error bars) of cells harvested from 13 healthy controls and 28 patients. *, $p < 0.05$; **, $p < 0.01$, Mann-Whitney U-test, two-sided.

cQTLs in autophagy-related genes influence *Borrelia*-induced cytokine production. Genetic factors are strongly influencing clinical disease outcome by having a large regulatory impact on cytokine production [12]. Therefore, we assessed genetic variability in autophagy genes that influence *Borrelia*-induced cytokine production. Cytokine quantitative trait loci (cQTL) analysis was performed as described previously [13]. Almost 200 SNPs in autophagy related genes were found to modulate *Borrelia*-induced cytokine production (Supplementary Figure 1) underlining our previous findings of the important functional correlation between autophagy and *Borrelia*-induced cytokine production. Two SNPs in ATG7 and ATG9B (rs73019544 and rs11760487) are shown exemplarily as suggestive cQTLs ($p < 0.05$) for *Borrelia*-induced IL-1β (Figure 4A+B). Figure 4C shows four SNPs with the most significant effects on *Borrelia*-induced innate cytokines as IL-1β and IL-6, Figure 4D shows the effect on adaptive cytokines as IL-17, IL-22 and IFNγ. To visualize the effect of different autophagy-related genes on *Borrelia*-induced cytokine production, a schematic overview of the autophagic machinery is shown in Figure 5, highlighting those genes modulating *Borrelia*-induced cytokine production, both innate- as well as adaptive-related cytokines.

A



B



C

Cytokines	Gene	SNP	p-value
IL-1β	ATG7	rs73019544	0,033
	ATG9B	rs11760487	0,038
	ATG9B	rs2566514	0,040
	ATG16L2	rs4944014	0,046
IL-6	EREG	rs1966937	0,007
	LAMP3	rs7427761	0,016
	ATG3	rs6772077	0,016
	ATG3	rs7625366	0,016

D

Cytokines	Gene	SNP	p-value
IL-17	WIPI-2	rs4724461	0,010
	ATG9B	rs13307588	0,016
	WIPI-2	rs4720532	0,017
	WIPI-2	rs4720533	0,017
IFN-γ	ATG3	rs73229382	0,004
	ATG3	rs17233960	0,004
	ATG3	rs17233925	0,004
	ATG9B	rs6944859	0,012
IL-22	ATG9B	rs11769256	0,010
	ATG9B	rs71539868	0,012
	ATG9B	rs10952299	0,013
	ATG9B	rs12703108	0,018

Figure 4. Genetic variants in autophagy genes are associated with *B. burgdorferi*-induced cytokine levels. Boxplots showing the association of genotypes at SNP (A) rs73019544 (p=0,033) and (B) rs11760487 (p=0,038) with *B. burgdorferi* induced IL-1β levels. The number of individuals per genotype is shown in parenthesis below each boxplot. (C-D) List of SNPs in autophagy genes which influence *B. burgdorferi*-induced cytokine production after (C) 24 hours and (D) 7 days (four most significant SNPs per cytokine).

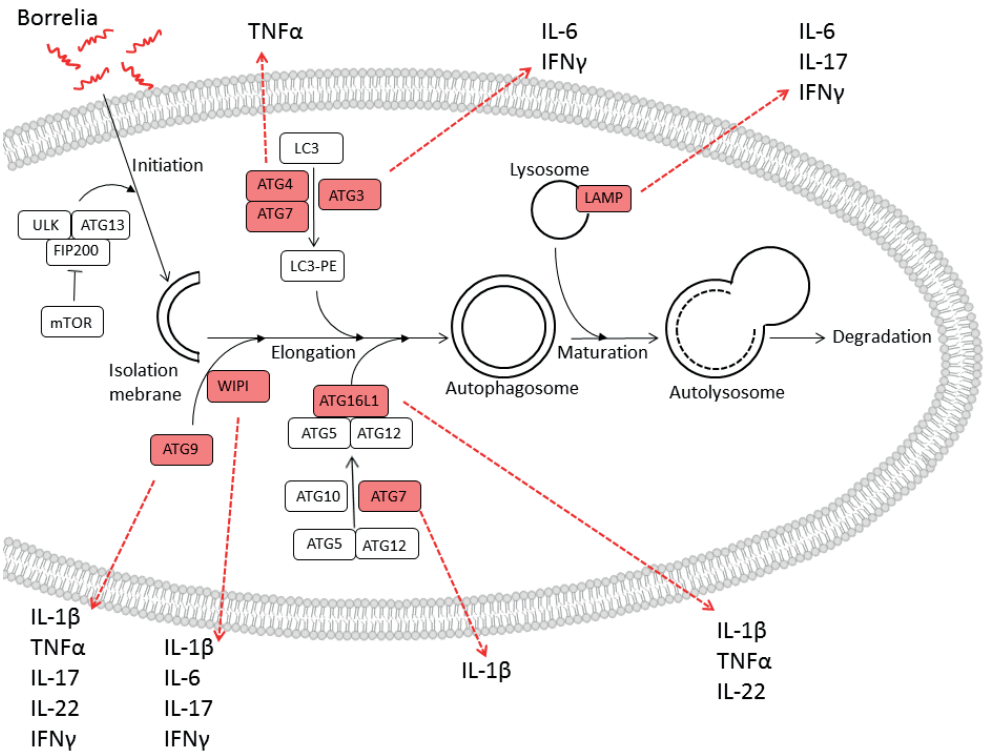


Figure 5. Schematic overview of autophagy genes influencing *B. burgdorferi*-induced cytokine responses. Initiation of autophagy begins with the formation of the isolation membrane. Further elongation and autophagosome completion requires different complexes of autophagy proteins, e.g. ATG5-ATG12-ATG16L-complex as well as the PE-conjugated microtubule-associated protein1 light chain 3 (LC3). SNPs in autophagy genes (indicated in red) have been shown to be responsible for differences in *B. burgdorferi*-induced cytokines.

Discussion

Lyme disease patients show a large diversity between their clinical symptoms ranging from distinctions in size and number of erythema migrans lesions up to secondary manifestations involving the central nervous systems, skin alterations and inflammatory joint swelling. Approximately 10 to 20% of patients report persistent symptoms lasting months to years [17]; why the other 80 % of patients don't suffer from continuing symptoms is not known. Although the connection between genetic variation and diverse cytokine responses leading to differences in clinical outcome has been made, little is known about the molecular pathways involved in this phenomenon.

In our approach, we designed a set of experiments assessing the transcriptional profile induced in circulating leukocytes by *B. burgdorferi*. Human primary PBMCs were stimulated either with RPMI or *Borrelia* spirochetes. Pathway analysis of genes induced by *Borrelia* indicated an upregulation of expected pathways involved in inflammation and innate immunity, but also a modulated expression of genes involved in autophagy. Previous studies have already described an altered cytokine profile of *Borrelia*-stimulated autophagy-incapable PBMCs [8,11]. To confirm this finding and to find the key players involved in this process, we looked into the expression of autophagy-related genes. Significant up- and downregulation of several autophagy-related genes as ATG7, ATG9 and ATG16L was found. Polymorphisms in ATG genes have been associated with autoimmune disorders as lupus erythematosus (SLE) [18], Crohn's diseases [19,20] and rheumatoid arthritis [21] indicating the clinical relevance of autophagy in immunological and inflammatory disorders.

To confirm these findings, we compared different expression profiles of autophagy-related genes in 29 Lyme disease patients to 13 matched controls. We identified several differentially expressed autophagy-related genes indicating a relevant role for those genes in the pathogenesis of the disease.

Analyzing the results, we found some autophagy-related genes to be upregulated, others downregulated in the process of the disease. Unfortunately, it is not known which modulated gene has the highest impact on the process of autophagy. It has been described that autophagy involves multiple molecular components, including the (1) ULK1 complex [22]; (2) the PI3K complex [23]; (3) transmembrane proteins as ATG9 [24]; (4) ubiquitin-like conjugation systems, involving ATG12 and LC3 [25]; as well as (5) several lysosomal hydrolases [26]. It is possible to speculate that the individual components of the autophagy machinery are differentially expressed depending on the time point of measurement. The downregulation of the autophagy suppressor mTOR, which is associated to the ULK1 complex, is in line with previous studies showing the induction of autophagy by *B. burgdorferi* stimulation [8,11]. ATG3 and ATG7, both upregulated in patients showing erythema migrans lesions, are components of the LC3 Conjugation System indicating as well an induction of autophagy by *B. burgdorferi* stimulation. ATG9 and ATG16L belong to different molecular components of the autophagy machinery, taking place on different time points which could explain their downregulation in Lyme disease patients at the moment of measurement.

Although most ATG proteins seem to be involved mainly in autophagy, some of them have been shown to exhibit nonautophagic functions as the participation in mitochondria homeostasis [27] or antiviral responses [28]. But the conclusion that autophagy is involved in the course of Lyme disease is not based on the regulation of one autophagy-related gene. Several autophagy genes are regulated by *B. burgdorferi* in our study; previous studies using pharmacological inhibitors of autophagy are also in line with our data [8,11].

Since cytokine responses are crucial in the pathophysiology of Lyme disease, we looked into the effect of autophagy genes on the production of cytokines. We have identified cQTLs in autophagy-related genes that strongly influence *Borrelia*-induced innate and adaptive cytokine production. Previous studies have already described the important role of IL-1β in the pathogenesis of Lyme disease. Early symptoms as low-grade fever and malaise can be mediated by this cytokine [4,29] and high amounts of this cytokine were found near the location of erythema migrans lesions after tick bites [30]. IL-1β induces in synergy with IL-23 the production of IL-17 and related cytokines from Th17 cells [10,31], which have been associated to increased joint damage in rheumatoid arthritis or psoriasis patients [7,32]. The role of adaptive cytokines such as IL-17 and IL-22 during the course of Lyme disease has not been fully understood yet, but there are some indications that both cytokines play a role in the persistent phase of the disease [33-35]. Single nucleotide polymorphisms (SNPs) in autophagy genes modulating the expression of those adaptive cytokines could be one explanation for the different disease outcome [36-38].

The incidence of Lyme disease has increased in the past few years [39], pointing to the urgency of new developments in treatment options. In our study, we have demonstrated a regulatory link between autophagy and *B. burgdorferi*-induced cytokines highlighting autophagy as a potential target for anti-inflammatory therapies in Lyme disease.

References

1. Steere AC, Coburn J, Glickstein L (2004) The emergence of Lyme disease. J Clin Invest 113: 1093-1101.

2. Berende A, ter Hofstede HJ, Vos FJ, van Middendorp H, Vogelaar ML, et al. (2016) Randomized Trial of Longer-Term Therapy for Symptoms Attributed to Lyme Disease. N Engl J Med 374: 1209-1220.

3. Balmelli T, Piffaretti JC (1995) Association between different clinical manifestations of Lyme disease and different species of Borrelia burgdorferi sensu lato. Res Microbiol 146: 329-340.

4. Miller LC, Isa S, Vannier E, Georgilis K, Steere AC, et al. (1992) Live Borrelia burgdorferi preferentially activate interleukin-1 beta gene expression and protein synthesis over the interleukin-1 receptor antagonist. J Clin Invest 90: 906-912.

5. Oosting M, van de Veerdonk FL, Kanneganti TD, Sturm P, Verschueren I, et al. (2011) Borrelia species induce inflammasome activation and IL-17 production through a caspase-1-dependent mechanism. Eur J Immunol 41: 172-181.

6. Oosting M, ter Hofstede H, van de Veerdonk FL, Sturm P, Kullberg BJ, et al. (2011) Role of interleukin-23 (IL-23) receptor signaling for IL-17 responses in human Lyme disease. Infect Immun 79: 4681-4687.

7. Geboes L, Dumoutier L, Kelchtermans H, Schurgers E, Mitera T, et al. (2009) Proinflammatory role of the Th17 cytokine interleukin-22 in collagen-induced arthritis in C57BL/6 mice. Arthritis Rheum 60: 390-395.

8. Buffen K, Oosting M, Mennens S, Anand PK, Plantinga TS, et al. (2013) Autophagy modulates Borrelia burgdorferi-induced production of interleukin-1beta (IL-1beta). J Biol Chem 288: 8658-8666.

9. Deretic V, Saitoh T, Akira S (2013) Autophagy in infection, inflammation and immunity. Nat Rev Immunol 13: 722-737.

10. Peral de Castro C, Jones SA, Ni Cheallaigh C, Hearnden CA, Williams L, et al. (2012) Autophagy regulates IL-23 secretion and innate T cell responses through effects on IL-1 secretion. J Immunol 189: 4144-4153.

11. Buffen K, Oosting M, Li Y, Kanneganti TD, Netea MG, et al. (2016) Autophagy suppresses host adaptive immune responses toward Borrelia burgdorferi. J Leukoc Biol.

12. Netea MG, van de Veerdonk FL, van der Meer JW (2012) Primary immunodeficiencies of pattern recognition receptors. J Intern Med 272: 517-527.

13. Li Y, Oosting, M., Deelen, P., Ricano-Ponce, I., Smeekens, S., Jaeger, M., Matzaraki, V., Swertz M.A., Xavier, R.J., Franke, L., Wijmenga C., Joosten, L.A.B., Kumar, V., Netea M.G. (2016) The architecture and variability of cytokine responses against bacterial and fungal human pathogens. Nature Medicine in press.

14. Delaneau O, Zagury JF, Marchini J (2013) Improved whole-chromosome phasing for disease and population genetic studies. Nat Methods 10: 5-6.

15. Howie B, Marchini J, Stephens M (2011) Genotype imputation with thousands of genomes. G3 (Bethesda) 1: 457-470.

16. Bindea G, Mlecnik B, Hackl H, Charoentong P, Tosolini M, et al. (2009) ClueGO: a Cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks. Bioinformatics 25: 1091-1093.

17. Bouquet J, Soloski MJ, Swei A, Cheadle C, Federman S, et al. (2016) Longitudinal Transcriptome Analysis Reveals a Sustained Differential Gene Expression Signature in Patients Treated for Acute Lyme Disease. MBio 7: e00100-00116.

18. Ramos PS, Criswell LA, Moser KL, Comeau ME, Williams AH, et al. (2011) A comprehensive analysis of shared loci between systemic lupus erythematosus (SLE) and sixteen autoimmune diseases reveals limited genetic overlap. PLoS Genet 7: e1002406.

19. Wellcome Trust Case Control C (2007) Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. Nature 447: 661-678.

20. Henckaerts L, Cleynen I, Brinar M, John JM, Van Steen K, et al. (2011) Genetic variation in the autophagy gene ULK1 and risk of Crohn's disease. Inflamm Bowel Dis 17: 1392-1397.

21. Raychaudhuri S, Thomson BP, Remmers EF, Eyre S, Hinks A, et al. (2009) Genetic variants at CD28, PRDM1 and CD2/CD58 are associated with rheumatoid arthritis risk. Nat Genet 41: 1313-1318.

22. Egan DF, Shackelford DB, Mihaylova MM, Gelino S, Kohnz RA, et al. (2011) Phosphorylation of ULK1 (hATG1) by AMP-activated protein kinase connects energy sensing to mitophagy. Science 331: 456-461.

23. He C, Levine B (2010) The Beclin 1 interactome. Curr Opin Cell Biol 22: 140-149.

24. Reggiori F, Klionsky DJ (2006) Atg9 sorting from mitochondria is impaired in early secretion and VFT-complex mutants in Saccharomyces cerevisiae. J Cell Sci 119: 2903-2911.

25. Mizushima N, Noda T, Yoshimori T, Tanaka Y, Ishii T, et al. (1998) A protein conjugation system essential for autophagy. Nature 395: 395-398.

26. Kroemer G, Jaattela M (2005) Lysosomes and autophagy in cell death control. Nat Rev Cancer 5: 886-897.

27. Radoshevich L, Murrow L, Chen N, Fernandez E, Roy S, et al. (2010) ATG12 conjugation to ATG3 regulates mitochondrial homeostasis and cell death. Cell 142: 590-600.

28. Hwang S, Maloney NS, Bruinsma MW, Goel G, Duan E, et al. (2012) Nondegradative role of Atg5-Atg12/ Atg16L1 autophagy protein complex in antiviral activity of interferon gamma. Cell Host Microbe 11: 397-409.

29. Habicht GS, Beck G, Benach JL, Coleman JL, Leichtling KD (1985) Lyme disease spirochetes induce human and murine interleukin 1 production. J Immunol 134: 3147-3154.

30. Jones KL, Muellegger RR, Means TK, Lee M, Glickstein LJ, et al. (2008) Higher mRNA levels of chemokines and cytokines associated with macrophage activation in erythema migrans skin lesions in patients from the United States than in patients from Austria with Lyme borreliosis. Clin Infect Dis 46: 85-92.

31. Mills KH, Dungan LS, Jones SA, Harris J (2013) The role of inflammasome-derived IL-1 in driving IL-17 responses. J Leukoc Biol 93: 489-497.

32. Ma HL, Liang S, Li J, Napierata L, Brown T, et al. (2008) IL-22 is required for Th17 cell-mediated pathology in a mouse model of psoriasis-like skin inflammation. J Clin Invest 118: 597-607.

33. Codolo G, Amedei A, Steere AC, Papinutto E, Cappon A, et al. (2008) Borrelia burgdorferi NapA-driven Th17 cell inflammation in lyme arthritis. Arthritis Rheum 58: 3609-3617.

34. Kotloski NJ, Nardelli DT, Peterson SH, Torrealba JR, Warner TF, et al. (2008) Interleukin-23 is required for development of arthritis in mice vaccinated and challenged with Borrelia species. Clin Vaccine Immunol 15: 1199-1207.

35. Bachmann M, Horn K, Rudloff I, Goren I, Holdener M, et al. (2010) Early production of IL-22 but not IL-17 by peripheral blood mononuclear cells exposed to live Borrelia burgdorferi: the role of monocytes and interleukin-1. PLoS Pathog 6: e1001144.

36. Plantinga TS, Crisan TO, Oosting M, van de Veerdonk FL, de Jong DJ, et al. (2011) Crohn's disease-associated ATG16L1 polymorphism modulates pro-inflammatory cytokine responses selectively upon activation of NOD2. Gut 60: 1229-1235.

37. Saitoh T, Fujita N, Jang MH, Uematsu S, Yang BG, et al. (2008) Loss of the autophagy protein Atg16L1 enhances endotoxin-induced IL-1beta production. Nature 456: 264-268.

38. Reed M, Morris SH, Owczarczyk AB, Lukacs NW (2015) Deficiency of autophagy protein Map1-LC3b mediates IL-17-dependent lung pathology during respiratory viral infection via ER stress-associated IL-1. Mucosal Immunol 8: 1118-1130.

39. Stanek G, Wormser GP, Gray J, Strle F (2012) Lyme borreliosis. Lancet 379: 461-473.

Additional files

Cytokines	SNP	Gene	p-value
IL-1β	rs2566511	ATG9B	0,030
	rs73019544	ATG7	0,033
	rs11760487	ATG9B	0,038
	rs2566514	ATG9B	0,040
	rs4944014	ATG16L2	0,046
	rs2909211	WIPI-1	0,047
	rs3862794	ATG16L2	0,048
	rs2952290	WIPI-1	0,050
IL-6	rs1966937	EREG	0,007
	rs7427761	LAMP3	0,016
	rs6772077	ATG3	0,016
	rs7625366	ATG3	0,016
	rs9809247	ATG3	0,016
	rs9848054	ATG3	0,016
	rs2279532	ATG3	0,016
	rs34233684	ATG3	0,016
	rs5000995	ATG3	0,016
	rs6792947	ATG3	0,016
	rs7652377	ATG3	0,016
	rs9869455	ATG3	0,016
	rs9869579	ATG3	0,016
	rs6762481	LAMP3	0,023
	rs9987030	WIPI-2	0,029
	rs564468	LAMP3	0,034
	rs6775326	LAMP3	0,035
	rs591614	LAMP3	0,035
	rs591618	LAMP3	0,035
	rs481410	LAMP3	0,035
	rs589445	LAMP3	0,035
	rs483150	LAMP3	0,035
	rs685585	LAMP3	0,035
	rs684703	LAMP3	0,035
	rs684708	LAMP3	0,035
	rs514636	LAMP3	0,035
	rs682396	LAMP3	0,035
	rs683395	LAMP3	0,035
	rs6762591	LAMP3	0,035
	rs6414495	LAMP3	0,035
	rs6414497	LAMP3	0,035
	rs6414498	LAMP3	0,035
	rs6414499	LAMP3	0,035
	rs6414500	LAMP3	0,035
	rs6414496	LAMP3	0,036
	rs625677	LAMP3	0,039
TNFα	rs4396149	ATG4C	0,027
	rs1981067	ATG4C	0,029
	rs11760487	ATG9B	0,030
	rs6587993	ATG4C	0,032
	rs6670072	ATG4C	0,032
	rs6676821	ATG4C	0,032
	rs6677051	ATG4C	0,032
	rs10493327	ATG4C	0,033
	rs11208045	ATG4C	0,033
	rs11208046	ATG4C	0,033

Cytokines	SNP	Gene	p-value
TNFα	rs11208049	ATG4C	0,033
	rs11208050	ATG4C	0,033
	rs11208051	ATG4C	0,033
	rs11208052	ATG4C	0,033
	rs11584363	ATG4C	0,033
	rs11803404	ATG4C	0,033
	rs12128286	ATG4C	0,033
	rs12567999	ATG4C	0,033
	rs12743698	ATG4C	0,033
	rs12748640	ATG4C	0,033
	rs12756081	ATG4C	0,033
	rs1333730	ATG4C	0,033
	rs1333736	ATG4C	0,033
	rs17097064	ATG4C	0,033
	rs1931079	ATG4C	0,033
	rs2886771	ATG4C	0,033
	rs3211074	ATG4C	0,033
	rs3737892	ATG4C	0,033
	rs3737893	ATG4C	0,033
	rs4448545	ATG4C	0,033
	rs59681313	ATG4C	0,033
	rs6587992	ATG4C	0,033
	rs6587996	ATG4C	0,033
	rs6587997	ATG4C	0,033
	rs6656119	ATG4C	0,033
	rs6670694	ATG4C	0,033
	rs6676637	ATG4C	0,033
	rs6683732	ATG4C	0,033
	rs6693530	ATG4C	0,033
	rs6695607	ATG4C	0,033
	rs7521483	ATG4C	0,033
	rs7540205	ATG4C	0,033
	rs7553110	ATG4C	0,033
	rs7555606	ATG4C	0,033
	rs998096	ATG4C	0,033
	rs10889385	ATG4C	0,033
	rs11208043	ATG4C	0,033
	rs7512374	ATG4C	0,033
	rs7527905	ATG4C	0,033
	rs7524508	ATG4C	0,033
	rs11587081	ATG4C	0,033
	rs7512529	ATG4C	0,033
	rs6587990	ATG4C	0,034
	rs6587991	ATG4C	0,034
	rs7519205	ATG4C	0,034
	rs11208040	ATG4C	0,034
	rs11208037	ATG4C	0,034
	rs11208038	ATG4C	0,034
	rs12134455	ATG4C	0,034
	rs2366820	ATG4C	0,034
	rs2366821	ATG4C	0,034
	rs6682286	ATG4C	0,034
	rs6657235	ATG4C	0,034
	rs6665932	ATG4C	0,034

Cytokines	SNP	Gene	p-value
TNFα	rs10749735	ATG4C	0,034
	rs6660873	ATG4C	0,034
	rs12120847	ATG4C	0,034
	rs6686232	ATG4C	0,034
	rs11208035	ATG4C	0,034
	rs11579518	ATG4C	0,034
	rs12752360	ATG4C	0,034
	rs7513520	ATG4C	0,035
	rs11208032	ATG4C	0,036
	rs6657707	ATG4C	0,037
	rs74669541	ATG16L2	0,044
	rs2184192	ATG4C	0,046
IL-17	rs4724461	WIPI-2	0,010
	rs13307588	ATG9B	0,016
	rs4720532	WIPI-2	0,017
	rs4720533	WIPI-2	0,017
	rs506385	LAMP3	0,021
	rs11761408	WIPI-2	0,023
	rs4236378	WIPI-2	0,026
	rs9987030	WIPI-2	0,026
	rs10266701	ATG9B	0,029
	rs677950	LAMP3	0,029
	rs4367443	WIPI-2	0,037
	rs4527412	LAMP3	0,037
	rs7646607	LAMP3	0,038
	rs4543037	LAMP3	0,038
	rs551488	LAMP3	0,039
	rs552648	LAMP3	0,039
	rs629478	LAMP3	0,039
	rs616035	LAMP3	0,041
	rs677952	LAMP3	0,041
	rs4538779	WIPI-2	0,042
	rs6971240	WIPI-2	0,042
	rs535658	LAMP3	0,043
	rs3801045	WIPI-2	0,043
	rs482912	LAMP3	0,043
	rs535651	LAMP3	0,044
	rs598252	LAMP3	0,044
	rs562013	LAMP3	0,044
	rs599011	LAMP3	0,044
	rs649355	LAMP3	0,045
	rs573813	LAMP3	0,046
	rs573940	LAMP3	0,046
	rs675924	LAMP3	0,046
	rs515818	LAMP3	0,046
	rs676290	LAMP3	0,046
	rs663739	LAMP3	0,046
	rs522235	LAMP3	0,048
	rs831256	LAMP3	0,048

Cytokines	SNP	Gene	p-value
IL-22	rs11769256	ATG9B	0,010
	rs71539868	ATG9B	0,012
	rs10952299	ATG9B	0,013
	rs12703108	ATG9B	0,018
	rs4944804	ATG16L2	0,032
	rs16851141	EREG	0,034
	rs11604683	ATG16L2	0,043
IFNγ	rs73229382	ATG3	0,004
	rs17233960	ATG3	0,004
	rs17233925	ATG3	0,004
	rs6944859	ATG9B	0,012
	rs34058562	WIPI-2	0,014
	rs10952299	ATG9B	0,017
	rs6947821	ATG9B	0,018
	rs11543123	LAMP3	0,018
	rs2303922	ATG9B	0,019
	rs13082005	ATG3	0,019
	rs491841	LAMP3	0,020
	rs574718	LAMP3	0,020
	rs2373927	ATG9B	0,021
	rs3772715	LAMP3	0,021
	rs3821520	LAMP3	0,025
	rs2279532	ATG3	0,025
	rs34233684	ATG3	0,025
	rs5000995	ATG3	0,025
	rs6792947	ATG3	0,025
	rs7652377	ATG3	0,025
	rs9869455	ATG3	0,025
	rs9869579	ATG3	0,025
	rs3772714	LAMP3	0,025
	rs6772077	ATG3	0,025
	rs7625366	ATG3	0,025
	rs9809247	ATG3	0,025
	rs9848054	ATG3	0,025
	rs3821517	LAMP3	0,025
	rs11718831	LAMP3	0,025
	rs11769256	ATG9B	0,028
	rs11975690	ATG9B	0,030
	rs2116023	ATG9B	0,038
	rs9987030	WIPI-2	0,039
	rs68044403	EREG	0,041

Supplementary figure 1. List of SNPs in autophagy genes which influence B. burgdorferi-induced cytokine production after 24 hours (IL-1β, IL-6, TNFα) and 7 days (IL-17, IL-22 and IFNγ).

Chapter 6

Autophagy Controls BCG-Induced Trained Immunity and the Response to Intravesical BCG Therapy for Bladder Cancer

PLoS Pathog. 2014 Oct 30;10(10):e1004485.

Kathrin Buffen, Marije Oosting, Jessica Quintin, Aylwin Ng, Johanneke Kleinnijenhuis, Vinod Kumar, Esther van de Vosse, Cisca Wijmenga, Reinout van Crevel, Egbert Oosterwijk, Anne J. Grotenhuis, Sita H. Vermeulen, Lambertus A. Kiemeney , Frank L. van de Veerdonk , Georgios Chamilos, Ramnik J. Xavier, Jos W. M. van der Meer, Mihai G. Netea, Leo A.B. Joosten

Abstract

The anti-tuberculosis-vaccine Bacillus Calmette-Guérin (BCG) is the most widely used vaccine in the world. In addition to its effects against tuberculosis, BCG vaccination also induces non-specific beneficial effects against certain forms of malignancy and against infections with unrelated pathogens. It has been recently proposed that the non-specific effects of BCG are mediated through epigenetic reprogramming of monocytes, a process called *trained immunity*. In the present study we demonstrate that autophagy contributes to trained immunity induced by BCG. Pharmacologic inhibition of autophagy blocked trained immunity induced *in vitro* by stimuli such as β–glucans or BCG. Single nucleotide polymorphisms (SNPs) in the autophagy genes *ATG2B* (rs3759601) and *ATG5* (rs2245214) influenced both the *in vitro* and *in vivo* training effect of BCG upon restimulation with unrelated bacterial or fungal stimuli. Furthermore, pharmacologic or genetic inhibition of autophagy blocked epigenetic reprogramming of monocytes at the level of H3K4 trimethylation. Finally, we demonstrate that rs3759601 in *ATG2B* correlates with progression and recurrence of bladder cancer after BCG intravesical instillation therapy. These findings identify a key role of autophagy for the nonspecific protective effects of BCG.

Author Summary

Next to its effects against tuberculosis, BCG vaccination also induces non-specific beneficial effects on immune cells to increase their ability to control unrelated pathogens. It has been recently proposed that the non-specific effects of BCG are mediated through epigenetic reprogramming of monocytes, a process called trained immunity. Little is known regarding the intracellular events controlling its induction. In this study we identified autophagy as a key player in trained immunity. Pharmacological inhibition of autophagy as well as polymorphisms in autophagy-related genes blocked BCG-induced trained immunity. Furthermore, BCG vaccine is also used to treat bladder cancer. Genetic polymorphisms in autophagy-related genes correlated with progression and recurrence of bladder cancer after treatment with BCG therapy. These findings open new possibilities for improvement of future BCG-based vaccines to be used against infections and malignancies.

Introduction

Immunological memory has long been viewed as being exclusively mediated by T and B cells. However, an increasing body of evidence indicates enhanced nonspecific protection against reinfections in plants [1] and insects [2] which lack adaptive immunity. Similarly, mammalian innate immune cells such as natural killer cells show features of immunological memory [3,4]. Recently, we proposed the term *trained immunity* to describe the memory properties of innate immune cells [5]. *Candida albicans* or its major cell wall component β-glucan, as well as BCG, are prominent stimuli that can induce trained immunity through epigenetic reprogramming of monocytes [6,7]. However, little is known regarding the intracellular events controlling the induction of trained immunity, impairing the ability to fully harness the therapeutic potential of this important immunological process. Therefore, we investigated the trained immunity-induced signaling pathways, discovering autophagy being one of the main players.

Results

β-glucan training induces the transcription of autophagy-related proteins. To identify new signaling pathways specifically activated upon training of monocytes with bacterial components, we compared the transcriptional profile of β-glucan-trained human primary monocytes isolated from healthy volunteers to the profile of monocytes stimulated with *Escherichia coli*-derived lipopolysaccharide (LPS), which stimulates inflammation but is unable to induce long-term training [5]. Transcriptomic assessment of these monocytes by microarrays and pathway analysis revealed specific clusters of genes significantly induced by β-glucan training with an intriguing signal found in the ubiquitin-related proteins and associated catabolic processes (Figure 1A). Since ubiquitination plays an important role in autophagy [8], a process that has previously been shown to improve intracellular processing of BCG [9,10], we examined the role of autophagy in the induction of trained immunity.

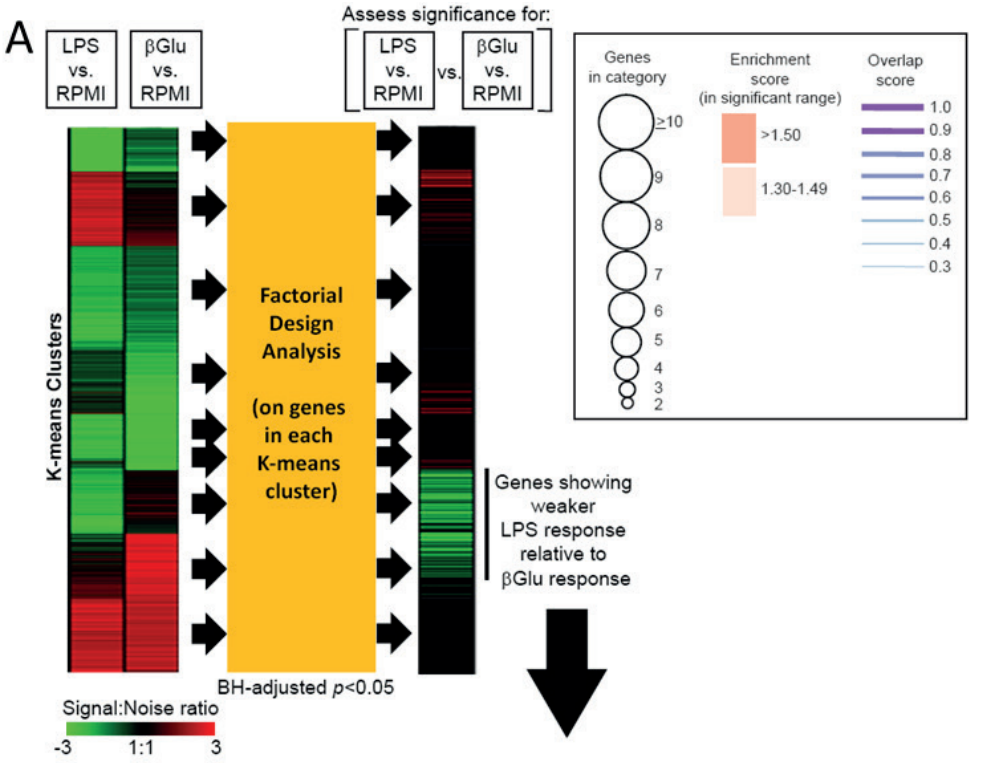
Autophagy is essential for β-glucan and BCG training in monocytes. Using an *in vitro* model of trained immunity [6,7], adherent monocytes from healthy human volunteers were stimulated for 24h with RPMI, BCG or β-glucan alone or in combination with the autophagy inhibitors 3-methyladenine (3MA) or wortmannin. After washing of cells and

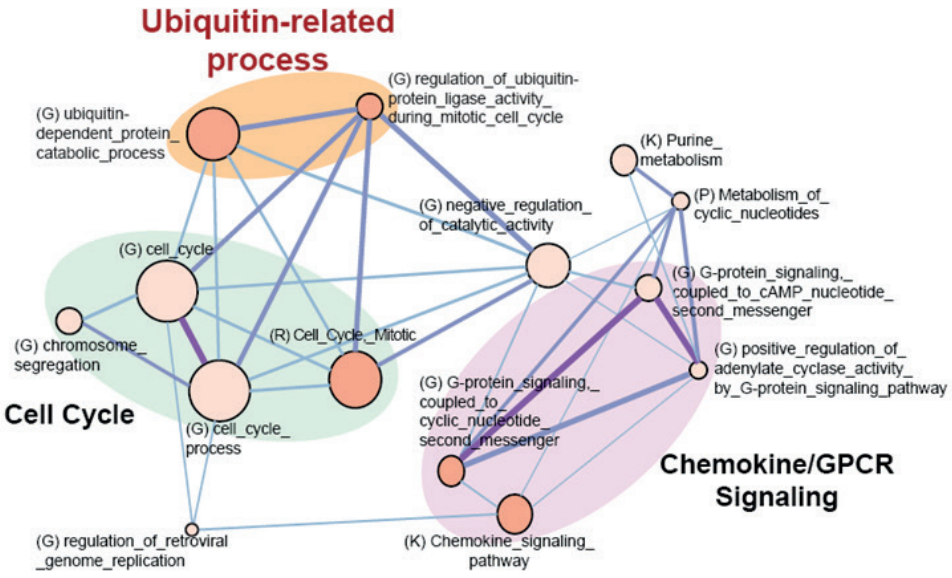
a resting period of 6 days in medium supplemented with 10% human serum, cytokine production was measured after a second stimulation with the unrelated stimuli LPS or *Borrelia burgdorferi* (*B. burgdorferi*) (Figure 1B). IL-6 and TNF-α production increased significantly in BCG- and β-glucan-trained cells compared to non-trained cells. When autophagy was blocked by 3MA or wortmannin, neither β–glucan nor BCG induced *trained immunity* (Figure 1C–F; Figure S1A-H). Notably, the putative cytotoxic effects of autophagy inhibitors used in this study were assessed by LDH measurements. None of the inhibitors used during the 24h of primary cell stimulation enhanced LDH release compared to RPMI-treated cells (Figure S2 A-C), demonstrating that the molecules were not toxic to the cells.

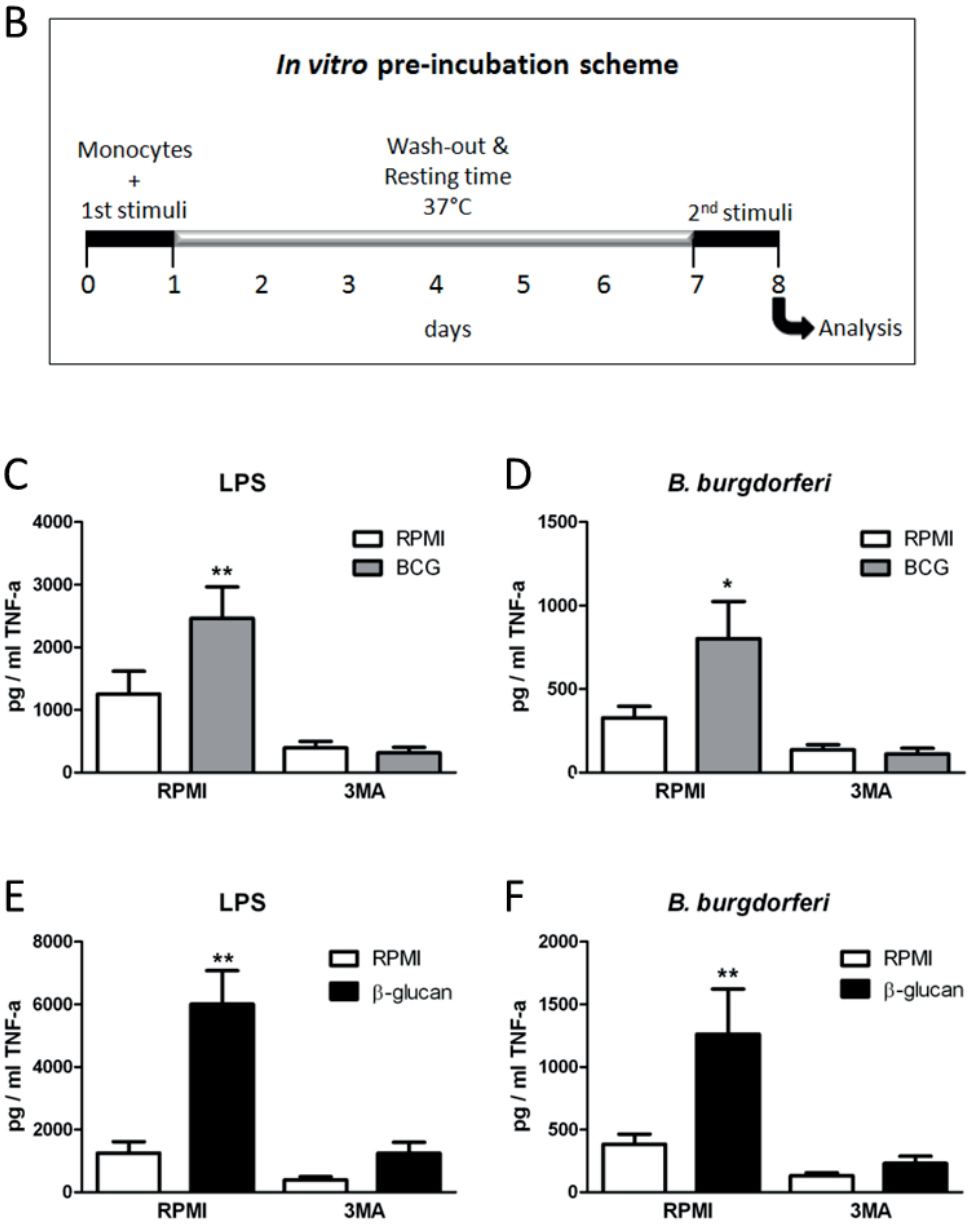
Single nucleotide polymorphisms in *ATG2B* and *ATG5* negatively influence trained immunity. To further explore the role of autophagy in the nonspecific protection of BCG in innate immune cells, we examined the effects of genetic polymorphisms in autophagy genes for the BCG-induced trained immunity *in vitro* and *in vivo*. The genotypes of nine SNPs in eight autophagy genes were correlated with the capacity of BCG to induce trained immunity in a group of 72 volunteers. The rs3759601 *ATG2B* SNP was found to be strongly associated with trained immunity; the ability to develop training characteristics following BCG treatment was observed in monocytes isolated from individuals carrying the GG (major) or CG genotype but not in those carrying the CC (minor) genotype (plus strand coding) (Figure 2A-F). A similar effect, though less clear, was apparent for the rs2245214 *ATG5* SNP (Figure 2G-I). No significant association was found between the nonspecific protection of BCG and polymorphisms in *ATG10, ATG16L1, EREG, IRGM, LAMP3* and *WIPI* (Figure S3).

page 107 + 108 →

Figure 1. Role of autophagy for the training of monocytes. (A) Transcriptome profiling and pathway analysis of β-glucan training of monocytes compared to LPS stimulation. Factorial design analysis was performed on genes in each K-means cluster to assess significance of response differences elicited by LPS and β-glucan (Benjamini-Hochberg (BH)-adjusted p < 0.05). The signal:noise ratio is shown as heatmaps. Functional enrichment (or molecular concept) map was generated for genes exhibiting significantly weaker LPS response relative to β-glucan response. This map summarizes the extent of mutual overlap between gene sets and identifies a cluster of strongly connected gene sets that are enriched among genes showing stronger β-glucan response. Only enriched gene sets in the significant range with gene set enrichment score (-Log10(p)>1.3; p<0.05) are shown. Nodes denote enriched gene sets or „annotation terms/categories", assembled from (K) KEGG pathways, (G) Gene Ontology, (P) Panther pathways, (R) Reactome. Node size corresponds to the number of gene members in each gene set. Node color denotes the gene set enrichment score. Please refer to graphical legend (boxed) in figure. The extent of mutually overlapping genes between gene sets is represented by thickness and color intensity of edges connecting nodes. The overlap score is the average of the Jaccard and Overlap coefficients. Strongly connected network components were identified using Tarjan's algorithm. Important ubiquitin-related processes in map are highlighted. (B) Diagram showing the course of the in vitro preincubation experiment. (C-F) BCG (C-D) or β-glucan (E-F) training in vitro in the presence or absence of 3MA using freshly isolated human monocytes and different stimuli for restimulation (LPS, *B. burgdorferi*). *P<0.05, **P<0.01.


A
LPS vs. RPMI
βGlu vs. RPMI
Assess significance for:
LPS vs. RPMI
vs.
βGlu vs. RPMI
Genes in category
Enrichment score (in significant range)
Overlap score
≥10
9
8
7
6
5
4
3
2
>1.50
1.30-1.49
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
K-means Clusters
Factorial Design Analysis
(on genes in each K-means cluster)
Genes showing weaker LPS response relative to βGlu response
BH-adjusted p<0.05
Signal:Noise ratio
-3
1:1
3


Ubiquitin-related process
(G) ubiquitin-dependent_protein_catabolic_process
(G) regulation_of_ubiquitin-protein_ligase_activity_during_mitotic_cell_cycle
(K) Purine_metabolism
(P) Metabolism_of_cyclic_nucleotides
(G) negative_regulation_of_catalytic_activity
(G) cell_cycle
(G) G-protein_signaling,_coupled_to_cAMP_nucleotide_second_messenger
(G) chromosome_segregation
(R) Cell_Cycle,_Mitotic
(G) positive_regulation_of_adenylate_cyclase_activity_by_G-protein_signaling_pathway
Cell Cycle
(G) cell_cycle_process
(G) G-protein_signaling,_coupled_to_cyclic_nucleotide_second_messenger
Chemokine/GPCR Signaling
(G) regulation_of_retroviral_genome_replication
(K) Chemokine_signaling_pathway



Continuation Figure 1
(legend on page 106)

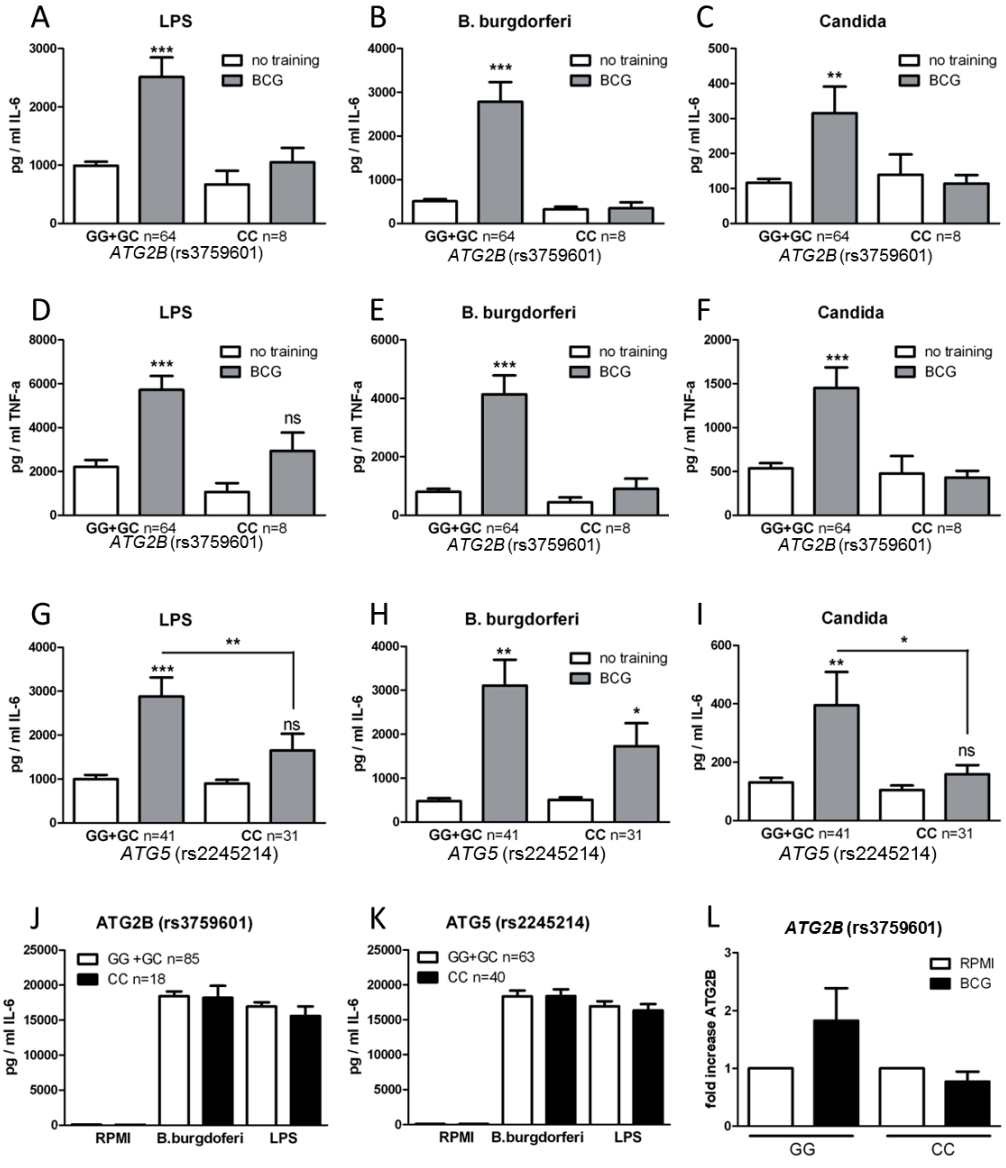


Figure 2. Polymorphisms in ATG2B or ATG5 diminish the training capacity of human monocytes. (A-I) Blood was collected from volunteers and genotyped for ATG2B rs3759601 (A-F) and ATG5 rs2245214 (G-I). Human monocytes were trained with BCG for 24 h, washed and incubated in RPMI (10% human serum) for 6 d, after which they were restimulated for 24h with a second stimulus (LPS, Bb, or C. albicans). Proinflammatory cytokine production (IL-6 and TNF-α) was assessed by ELISA in the supernatants. (J-K) PBMCs isolated from volunteers carrying different genotypes for SNPs rs3759601 or rs2245214 were stimulated for 24 h with LPS or *B. burgdorferi*. IL-6 was measured in the supernatants by ELISA. (L) Human monocytes carrying different genotypes for SNP rs3759601 were trained with BCG for 4h. Expression of ATG2B was assessed by qPCR *P<0.05, **P<0.01.

To test the possibility that the association between SNPs and differences in cytokine production of BCG-trained monocytes was due to differential intrinsic capacity of the cells to produce cytokines, we stimulated monocytes bearing different *ATG2B* (Figure 2J) or *ATG5* (Figure 2K) alleles with LPS or *B. burgdorferi* for 24 hours. We noted no differences in cytokine release, indicating that the capacity of cells to release proinflammatory cytokines upon stimulation was not responsible for the observed association between autophagy SNPs and BCG-induced trained immunity. Next to that, the effect of the rs3759601 SNP on the transcription of the *ATG2B* gene was assessed after training. We observed increased levels of *ATG2B* transcripts in BCG-trained cells of individuals carrying the GG genotype but not in those carrying the CC genotype (Figure 2L). Increased *ATG2B* levels could also be found in β-glucan trained individuals carrying the GG genotype (Figure S4A) but no difference in *ATG2B* levels could be found in the two groups after LPS stimulation (Figure S4B). The reduced expression of *ATG2B* in individuals carrying the CC genotype of the SNP upon training with BCG could indicate a role for autophagy in trained immunity since it has been shown that the ATG2 proteins are essential for the formation of autophagosomes [11].

Autophagy is influenced by *ATG2B* single nucleotide polymorphism. To identify the effect of rs3759601 in *ATG2B* on autophagy, the amount of LC3+ vesicles in BCG stimulated monocytes of individuals carrying the major or minor variant of the SNP have been compared. A decrease in autophagosome formation of individuals carrying the CC genotype can be seen as demonstrated by a lower percentage of LC3+ monocytes (Figure 3A-B).

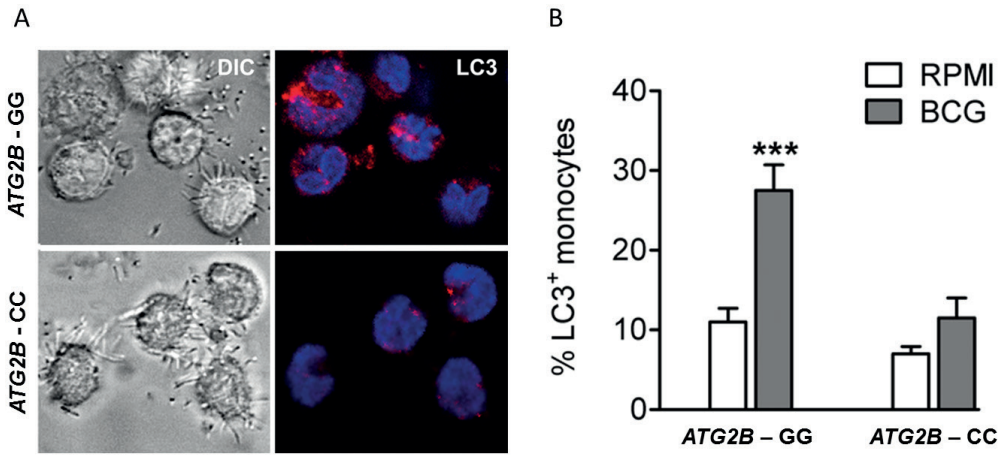


Figure 3. Autophagy affected by SNP in ATG2B. (A-B)Monocytes genotyped for ATG2B rs3759601 were seeded on coverslips, and stimulated with BCG. After 1 hour of stimulation, cells were fixed and stained with an antibody against LC3.

***ATG2B* single nucleotide polymorphism influences in vivo training of monocytes.** To corroborate the above data, we investigated BCG-induced training of monocytes *in vivo* by testing individuals carrying different *ATG2B* alleles. Monocytes were isolated from 16 healthy volunteers, before and 3 months after vaccination with BCG. Following stimulation with LPS (Figure S5A-B) or *B. burgdorferi* (Figure 4A-B), IL-1β and TNF-α production was significantly higher 3 months after vaccination in individuals who were bearing at least one G allele of the *ATG2B* SNP (n=12), while monocytes isolated from individuals carrying the CC genotype (n=4) showed no change in cytokine production after BCG vaccination.

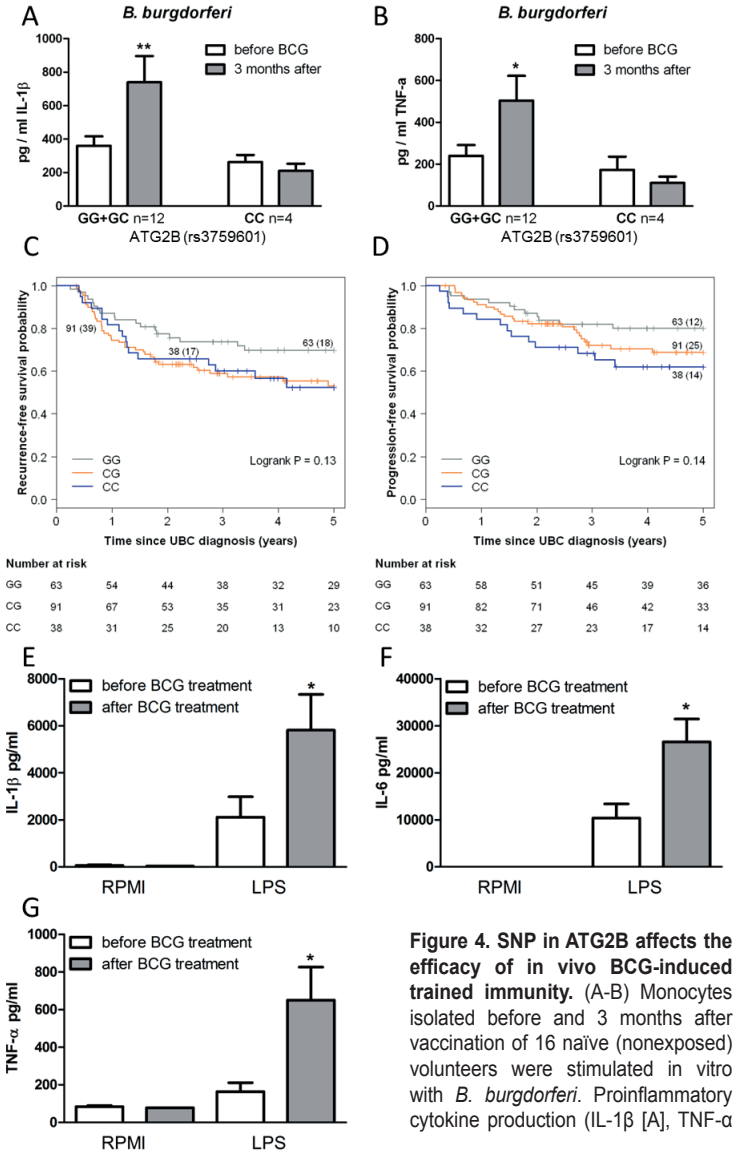


Figure 4. SNP in ATG2B affects the efficacy of in vivo BCG-induced trained immunity. (A-B) Monocytes isolated before and 3 months after vaccination of 16 naïve (nonexposed) volunteers were stimulated in vitro with *B. burgdorferi*. Proinflammatory cytokine production (IL-1β [A], TNF-α [B]) was assessed by ELISA in the supernatants. (C-D) Kaplan-Meier curves for recurrence-free (C) and progression-free (D) survival according to rs3759601 SNP genotype of 192 patients suffering from non-muscle invasive bladder cancer treated with ≥6 intravesical instillations of BCG. Each drop in a probability curve indicates one or more events in that group. Vertical lines indicate censored patients, i.e. those who reached the end of their follow-up without experiencing the event. Total number of patients and number of events (between brackets) per genotype category are indicated next to the corresponding curve. Numbers of patients at risk at selected time points for each genotype category are given below the plots. (E-G) Monocytes of bladder cancer patients isolated before and after 6 intravesical BCG instillations as initial treatment were stimulated in vitro with LPS. Proinflammatory cytokine production (IL-1β [E], IL-6 [F], TNF-α [G]) was assessed by ELISA in the supernatants *P<0.05, **P<0.01.

SNP in *ATG2B* correlates with the progression and recurrence of bladder cancer after BCG intravesical instillation therapy. In addition to the protective effects of BCG against secondary infections, non-specific therapy with intravesical BCG is also used as a therapeutic strategy for patients with non-muscle invasive bladder cancer (NMIBC; stages: Ta, T1, CIS) [12]. In a cohort of 192 NMIBC patients treated with at least 6 intravesical instillations of BCG we evaluated the association between the *ATG2B* SNP and prognosis in terms of recurrence and progression during the first five years after the primary NMIBC diagnosis. Analyses learned that those patients that carry one or two C alleles for *ATG2B* rs3759601 showed increased risk of recurrence (CG vs. GG: hazard ratio (HR) = 1.73 (95% confidence interval (CI): 0.99-3.03) and CC vs. GG: HR = 1.68 (95% CI: 0.78-3.27)) (Figure 4C) and progression (CG vs. GG: HR = 1.57 (95% CI: 0.79-3.12) and CC vs. GG: HR = 2.15 (95% CI: 1.00-4.66)) (Figure 4D). This finding of a correlation between the polymorphism in *ATG2B* to progression and recurrence of bladder cancer supports the hypothesis of a clinical relevance of the autophagy gene for the non-specific protective effects exerted by BCG. In addition, the responsiveness of circulating monocytes of bladder cancer patients has been investigated before and after BCG-therapy. Of high interest, individuals who received intravesical BCG therapy showed an increased cytokine response of their monocytes after stimulation with LPS *in vitro* (Figure 4E-G).

Pharmacologic or genetic inhibition of autophagy blocks epigenetic reprogramming of monocytes in response to BCG training. Epigenetic reprogramming of monocytes is a crucial immunological mechanism underlying nonspecific protection by BCG. Stable changes in histone trimethylation at the level of lysine 4 of histone 3 (H3K4), a post-translational modification associated with the regulation of immune-related genes [13], is one of the mechanisms responsible for enhanced cytokine production after re-stimulation of trained monocytes [5-7]. Therefore, we assessed whether trimethylation of H3K4 due to nonspecific training by BCG was influenced by the *ATG2B* polymorphism or inhibition of autophagy by 3MA. Consistent with our hypothesis, H3K4 trimethylation was significantly increased at the IL-6 and TNF-α promoters in BCG-trained monocytes from volunteers bearing the *ATG2B* G allele (Figure 5A-B). In contrast, volunteers homozygous for the *ATG2B* C allele did not show any increase in trimethylation at H3K4 at the cytokine promoters after BCG-training. Furthermore, inhibition of autophagy by 3MA blocked the H3K4 trimethylation at IL-6 and TNF-α promoters in BCG-trained monocytes (Figure 5C-D), supporting the hypothesis of a central role of autophagy in the epigenetic reprogramming of monocytes induced by BCG.

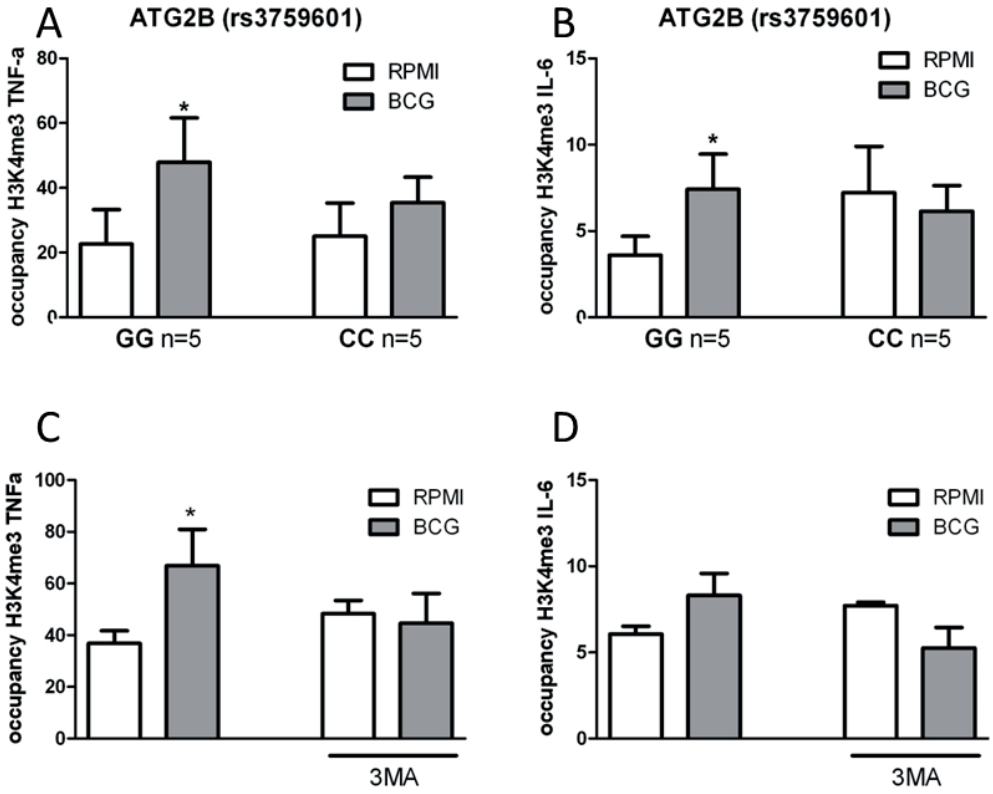


Figure 5. Impairment of autophagy decreases trimethylation at H3K4 in human monocytes. ChIP analysis of the enrichment of H3K4me3 at the promoter of (A) TNF-α and (B) IL-6 in human monocytes isolated from volunteers carrying the major variant (GG) or minor variant (CC) alleles for ATG2B after training with BCG. ChIP analysis of the enrichment of H3K4me3 at the promoter of (C) TNF-α and (D) IL-6 in human monocytes trained with BCG in the presence or absence of 3MA *p<0.05, **p<0.01.

Discussion

BCG is a live attenuated vaccine which is routinely administered at birth in low-income countries, protecting newborns against disseminated tuberculosis and tuberculosis meningitis [14]. However, in addition to its specific protection against childhood tuberculosis, epidemiological studies have demonstrated that BCG protects against infant mortality independent of its effect on tuberculosis, suggesting a nonspecific protection against unrelated infections [15-24]. Next to that, BCG treatment has long been used as a non-specific immunostimulatory therapy in urothelial cell carcinomas [25]. Recently, these non-specific protective mechanisms of BCG have been associated with epigenetic reprogramming of innate immune cells in a process called *trained immunity* [7]. In the

present study we show that autophagy is a central event modulating trained immunity induced by BCG. Moreover, polymorphisms in autophagy genes such as *ATG2B* control trained immunity in both *in vitro* and *in vivo* models, as well as the non-specific therapeutic effects of BCG in patients with bladder cancer.

An important difference has to be noted between the effect of *ATG2B* polymorphism on BCG training against secondary infections and BCG used as a treatment against non-muscle invasive bladder cancer. BCG training of monocytes against unrelated secondary infections could only be modulated by an *ATG2B* polymorphism expressed on both alleles. Heterozygote individuals were still trainable with the vaccine. On the contrary, the prognosis in terms of recurrence and progression of non-muscle invasive bladder cancer decreased with only one affected allele. The different route of BCG administration, as well as several disease-related mechanisms could be the explanation of this event. To further unravel the different mechanisms behind this phenomenon, a pilot study has been performed to investigate whether BCG installation in the bladder could induce a state of trained immunity. The cytokine response of ex-vivo stimulated monocytes of BCG treated bladder cancer patients increased in response to LPS compared to the pre-treatment response.

In addition to the aspects discussed above, there are also a few limitations of the current study. Thus, although we demonstrate the role of autophagy for BCG-induced trained immunity, additional studies are needed to decipher the precise pathway linking autophagy to the epigenetic modifications observed during trained immunity. A second important aspect is the fact that the genetic study has been performed in a relatively small cohort of patients with bladder carcinoma, and it needs to be validated by independent studies. Finally, the role of autophagy gene SNPs for the effects of BCG on infections also needs to be evaluated. The role of BCG for protection against infection is currently investigated by a large Danish study in 4500 newborn children (http://calmette-studiet.dk/), and the effect of the autophagy polymorphisms on the effects of BCG is an important aspect to be assessed.

A key question regarding trained immunity refers to the signaling and molecular mechanisms responsible for its induction. As shown previously, exposure of monoctyes to BCG induces high levels of H3K4 trimethylation at the promoter level of inflammatory genes, which correlates with long-term increased production of proinflammatory

cytokines, a hallmark of trained immunity [6,7]. Next to that, the blockage of histone acetyltransferases inhibits the training of monoctyes [26] suggesting also an important role of acetylation in trained immunity which will be further studied in the future.

The discovery that autophagy modulates trained immunity may have important consequences. It provides understanding of an important immunological process, although future studies are warranted to identify the molecular mechanisms through which autophagy mediates the epigenetic changes responsible for trained immunity. Restriction of reactive oxygen species release from damaged mitochondria, or processing of microbial ligands such as peptidoglycans [9], may represent two potential candidate mechanisms. Furthermore, identification of autophagy as a driver of trained immunity opens new possibilities for improvement of future BCG-based vaccines to be used against infections and malignancies.

Materials and Methods

Ethics statement. All human experiments were conducted according to the principles expressed in the Declaration of Helsinki. Before taking blood, informed written consent of each human subject was obtained. The study was approved by the review board of the department of Medicine of the Radboud University Nijmegen Medical Centre. The BCG *in vivo* study was approved by the Arnhem-Nijmegen Ethical Committee. For the NBCS, all participants gave written informed consent and the study was approved by the Institutional Review Board of the RUMC. All data analyzed were anonymized.

Healthy volunteers. *In vitro* cytokine stimulation experiments were performed with PBMCs isolated from buffy coats obtained from healthy volunteers (Sanquin Bloodbank, Nijmegen, the Netherlands). To analyze the effect of gene polymorphisms on trained immunity, blood was drawn from a group of healthy volunteers (age 23-73). For the *in vivo* BCG model, subjects (aged 20-36) who were scheduled to receive a BCG vaccination at the public health service, due to travel or work in tuberculosis-endemic countries, were asked to participate in this trial. Blood was drawn before and 3 months after the BCG vaccination. Informed consent was obtained from all human subjects.
The bladder cancer patients included in this study were selected from a total of 1,602 patients with primary urinary bladder cancer (UBC) from the Nijmegen Bladder Cancer

Study (NBCS). The NBCS served as the Dutch discovery population in the UBC genome-wide association study led by Radboud University Medical Centre (RUMC, Nijmegen, the Netherlands) and deCODE Genetics (Reykjavik, Iceland). The NBCS has been described in detail before [27]. Cases with a previous or simultaneous diagnosis of upper urinary tract cancer, based on information from the Netherlands Cancer Registry, were excluded. Detailed clinical data concerning diagnosis, stage, treatment, and disease course (tumor recurrence and progression) were collected retrospectively based on a medical file survey. In the analysis we included a total of 192 cases with non-muscle invasive bladder cancer (NMIBC; stage Ta/T1/CIS) that received at least 6 intravesical BCG instillations as initial treatment (median follow-up time from initial transurethral resection of bladder tumor until last urological check-up visit was 5.2 years (range: 0.4 - 20)). All patients were from Caucasian background.

Microorganisms. *C. albicans* ATCC MYA-3573 (UC 820) yeast was heat-inactivated for 30 min at 95°C. *B. burgdorferi*, ATCC strain 35210, was cultured at 33°C in Barbour-Stoenner-Kelley (BSK)-H medium (Sigma-Aldrich) supplemented with 6% rabbit serum. Spirochetes were grown to late-logarithmic phase and examined for motility by dark-field microscopy. Bacteria were harvested by centrifugation of the culture at 7000 x g for 15 min and washed twice with sterile PBS (pH 7.4).

Stimulation experiments. The mononuclear cell fraction was obtained by density centrifugation of blood diluted 1:1 in pyrogen-free saline over Ficoll-Paque (Pharmacia Biotech, Pittsburgh, Pennsylvania, USA). Cells were washed twice in saline and resuspended in culture medium (RPMI; Invitrogen, Carlsbad, California, USA) supplemented with 50 mg/L gentamicin, 2 mM L-glutamine and 1 mM pyruvate. PBMCs were counted in a Coulter counter (Coulter Electronics, Brea, California, USA) and their number was adjusted to 5 x 10^6 cells / ml. A total of 5 x 10^5 cells in a 100 µl volume was added to round-bottom 96-well plates (Greiner) with RPMI, *E. coli* LPS (10 ng/ml) or *B. burgdorferi* (1 x 10^6 /ml). After 24h, the supernatants were collected and stored at -20°C until being assayed.
For training experiments, PBMCs (5 x 10^5 for cytokine analysis; 10 x 10^6 for ChIP analysis) were incubated for 1 h at 37°C in 5% CO2. Adherent monocytes were selected by washing out nonadherent cells with warm PBS. Thereafter, cells were preincubated with RPMI, BCG vaccine (1 µg/ml BCG vaccine SSI from the Netherlands Vaccine Institute) or β-1,3-(D)-glucan (β-glucan) (10 ng/ml; kindly provided by Professor David Williams) for 24 h (4h for Real-time PCR). After a resting period of 6 d in RPMI including 10% serum, cells

were stimulated with *E. coli* LPS (10 ng/ml), *C. albicans* (1 x 10^6 /ml), *B. burgdorferi* (1 x 10^6 /ml), or RPMI for an additional 24h. Supernatants were stored at -20°C until ELISA was performed. In the "inhibition" experiments, before training with BCG or β-glucan, the adherent monocytes were preincubated for 1 h with 10mM 3-methyl adenine (3MA, Sigma).

Cytokine measurements. Concentrations of human IL-1β, IL-6 and TNF-α were determined in duplicates using commercial ELISA kits (Sanquin, Amsterdam, or R&D Systems, Minneapolis), in accordance with the manufacturers' instructions.

Real-time PCR. RNA from stimulated monocytes was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Isolated RNA was reverse-transcribed into complementary DNA using iScript cDNA synthesis kit (Bio-Rad). Quantitative real-time PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems) using a 7300 Real-time PCR system (Applied Biosystems). In each PCR a melting curve analysis was included to control for a specific PCR amplification. Primers used for the experiments (final concentration 10 μM) are shown below. Real-time quantitative PCR data were corrected for expression of the housekeeping gene *B2M*. Human *ATG2B* forward: ACCAGAGATAGCACCTTCTGAC and reverse: CCAATTAACCGTCCAATCTG; human *B2M* forward: ATGAGTATGCCTGCCGTGTG and reverse: CCAAATGCGGCATCTTCAAAC.

Isolation of genomic DNA and single nucleotide polymorphism analysis. *In vitro* training experiment: Using NCBI SNP database we selected SNPs in autophagy genes previously associated to diseases or with a minor allele frequency of at least 5% (*ATG10* (rs1864183), *ATG10* (rs3734114), *ATG16L1* (rs2241880), *ATG2B* (rs3759601) [allele frequency: G = 70%; C = 30%], *ATG5* (rs2245214), *EREG* (rs78803121), *IRGM* (rs4958847), *LAMP3* (rs482912), *WIPI* (rs883541)). Blood samples were obtained by venapuncture. Genomic DNA was isolated from EDTA blood using standard methods, and 5 ng of DNA was used for genotyping. Multiplex assays were designed using Mass ARRAY Designer Software (Sequenom) and genotypes were determined using Sequenom MALDI-TOF MS according to manufacturer's instructions (Sequenom Inc., San Diego, CA, USA) as described previously [28].
In vivo BCG-cohort: DNA was isolated using the Gentra Pure Gene Blood kit (Qiagen), in accordance with the manufacturer's protocol for whole blood. DNA was dissolved in a

final volume of 100 µl buffer. Genotyping of single nucleotide polymorphisms (SNPs) was performed using a pre-designed TaqMan H SNP genotyping assay (Applied Biosystems) according to the manufacturer's protocol.
NBCS: All bladder cancer patients were genotyped using the Illumina Infinium HumanCNV370-duo Bead-Chips. Imputation was performed (IMPUTE version 2.1 software) using the 1000 Genomes low-coverage pilot haplotypes (released June 2010, 120 chromosomes) and the HapMap3 haplotypes (released February 2009, 1920 chromosomes) as a combined reference panel[27]. SNP rs3759601 was imputed with IMPUTE info_score 0.99. The SNP followed Hardy-Weinberg equilibrium.

Transcriptome analysis. Gene expression was performed as described previously[29] and assessed using Illumina Human HT-12 Expression BeadChip according to manufacturer's instructions. The Illumina LIMS platform, BeadStudio was employed to perform image analysis, bead-level processing, and quantile normalization of array data.

Chromatin immunoprecipitation. Adherent monocytes were cultured as described above (see *Stimulation Experiments*). ChIP was performed using antibodies against H3K4me3 (Diagenode). ChIPed DNA was processed further for qPCR analysis. The following primers were used in the reaction (5'-3'): TNF-α forward: CAGGCAGGTTCTCTTCCTCT, TNF-α reverse: GCTTTCAGTGCTCATGGTGT; IL-6 forward: TCGTGCATGACTTCAGCTTT, IL-6 reverse: GCGCTAAGAAGCAGAACCAC; myoglobin forward: AGCATGGTGCCACTGTGCT, myoglobin reverse: GGCTTAATCTCTGCCTCATGAT.

Immunofluorescence staining. For immunofluorescence imaging, monocytes were seeded on coverslips pretreated with polylysine, fixed with 4% PFA for 15 min at room temperature followed by 10 min of fixation with ice-cold methanol at -20°C. After two washing steps with PBS, cells were permeabilized by 0.1% saponin (Sigma-Aldrich), blocked for 30 min in PBS plus 2% BSA, incubated for 1 h with a mouse mAb to LC3 (1:50; Nanotools), washed twice in PBS plus 2% BSA and stained by a secondary Alexa Fluor 555 goat anti-mouse Ab (1:500; Molecular Probes), followed by DNA staining with 10µM TO-PRO-3 iodide (642/661; Invitrogen). After the washing steps, slides were mounted in Prolong Gold antifade media (Molecular Probes). Images were acquired using a laser-scanning spectral confocal microscope (TCS SP2; Leica Microsystems) and LCS Lite software (Leica microsystems). 2 fields / donor including at least 40 cells each were counted and compared for the amount of LC3.

Statistical analysis. Data are expressed as mean ± SEM unless mentioned otherwise. Differences between experimental groups were tested using the non-parametrical two-sided Mann-Whitney *U* test (no normal distribution of measured cytokines); differences between multiple time points within one group (before versus after treatment) were tested using the Wilcoxon matched pair test (unless stated otherwise) performed on GraphPad Prism 4.0 software (GraphPad). *P* values of ≤0.05 were considered statistically significant. Kaplan-Meier survival and Cox proportional hazard regression analyses were performed to evaluate the association between rs3759601 and recurrence- and progression-free survival. Log-rank tests were calculated to compare survival curves between genotype categories. Imputed genotype probabilities were transformed to hard genotype calls based on a probability threshold of > 0.90. Statistical analyses were performed using IBM SPSS Statistics for Windows 20 (IBM Corp., Armonk, NY, USA) and survival plots were drawn using R software v3.0.2 (package 'survival') (R Development Core Team, Vienna, Austria).

Acknowledgements

We thank Mathieu Platteel for the DNA isolation for the gene expression experiments.

References

1. Durrant WE, Dong X (2004) Systemic acquired resistance. Annu Rev Phytopathol 42: 185-209.

2. Pham LN, Dionne MS, Shirasu-Hiza M, Schneider DS (2007) A specific primed immune response in Drosophila is dependent on phagocytes. PLoS Pathog 3: e26.

3. Sun JC, Beilke JN, Lanier LL (2009) Adaptive immune features of natural killer cells. Nature 457: 557-561.

4. O'Leary JG, Goodarzi M, Drayton DL, von Andrian UH (2006) T cell- and B cell-independent adaptive immunity mediated by natural killer cells. Nat Immunol 7: 507-516.

5. Netea MG, Quintin J, van der Meer JW (2011) Trained immunity: a memory for innate host defense. Cell Host Microbe 9: 355-361.

6. Quintin J, Saeed S, Martens JH, Giamarellos-Bourboulis EJ, Ifrim DC, et al. (2012) Candida albicans infection affords protection against reinfection via functional reprogramming of monocytes. Cell Host Microbe 12: 223-232.

7. Kleinnijenhuis J, Quintin J, Preijers F, Joosten LA, Ifrim DC, et al. (2012) Bacille Calmette-Guerin induces NOD2-dependent nonspecific protection from reinfection via epigenetic reprogramming of monocytes. Proc Natl Acad Sci U S A 109: 17537-17542.

8. Husnjak K, Dikic I (2012) Ubiquitin-binding proteins: decoders of ubiquitin-mediated cellular functions. Annu Rev Biochem 81: 291-322.

9. Jagannath C, Lindsey DR, Dhandayuthapani S, Xu Y, Hunter RL, Jr., et al. (2009) Autophagy enhances the efficacy of BCG vaccine by increasing peptide presentation in mouse dendritic cells. Nat Med 15: 267-276.

10. Min Y, Xu W, Liu D, Shen S, Lu Y, et al. (2010) Autophagy promotes BCG-induced maturation of human dendritic cells. Acta Biochim Biophys Sin (Shanghai) 42: 177-182.

11. Velikkakath AK, Nishimura T, Oita E, Ishihara N, Mizushima N (2012) Mammalian Atg2 proteins are essential for autophagosome formation and important for regulation of size and distribution of lipid droplets. Mol Biol Cell 23: 896-909.

12. Kawai K, Miyazaki J, Joraku A, Nishiyama H, Akaza H (2013) Bacillus Calmette-Guerin (BCG) immunotherapy for bladder cancer: current understanding and perspectives on engineered BCG vaccine. Cancer Sci 104: 22-27.

13. Foster SL, Hargreaves DC, Medzhitov R (2007) Gene-specific control of inflammation by TLR-induced chromatin modifications. Nature 447: 972-978.

14. Trunz BB, Fine P, Dye C (2006) Effect of BCG vaccination on childhood tuberculous meningitis and miliary tuberculosis worldwide: a meta-analysis and assessment of cost-effectiveness. Lancet 367: 1173-1180.

15. Levine MI, Sackett MF (1946) Results of BCG immunization in New York City. Am Rev Tuberc 53: 517-532.

16. Aronson JD (1948) Protective vaccination against tuberculosis, with special reference to BCG vaccine. Minn Med 31: 1336.

17. Ferguson RG, Simes AB (1949) BCG vaccination of Indian infants in Saskatchewan. Tubercle 30: 5-11.

18. Velema JP, Alihonou EM, Gandaho T, Hounye FH (1991) Childhood mortality among users and non-users of primary health care in a rural west African community. Int J Epidemiol 20: 474-479.

19. Niobey FM, Duchiade MP, Vasconcelos AG, de Carvalho ML, Leal Mdo C, et al. (1992) [Risk factors for death caused by pneumonia in children younger than 1 year old in a

metropolitan region of southeastern Brazil. A case- control study]. Rev Saude Publica 26: 229-238.

20. Kristensen I, Aaby P, Jensen H (2000) Routine vaccinations and child survival: follow up study in Guinea-Bissau, West Africa. BMJ 321: 1435-1438.

21. Garly ML, Martins CL, Bale C, Balde MA, Hedegaard KL, et al. (2003) BCG scar and positive tuberculin reaction associated with reduced child mortality in West Africa. A non-specific beneficial effect of BCG? Vaccine 21: 2782-2790.

22. Vaugelade J, Pinchinat S, Guiella G, Elguero E, Simondon F (2004) Non-specific effects of vaccination on child survival: prospective cohort study in Burkina Faso. BMJ 329: 1309.

23. Aaby P, Roth A, Ravn H, Napirna BM, Rodrigues A, et al. (2011) Randomized trial of BCG vaccination at birth to low-birth-weight children: beneficial nonspecific effects in the neonatal period? J Infect Dis 204: 245-252.

24. Biering-Sorensen S, Aaby P, Napirna BM, Roth A, Ravn H, et al. (2012) Small randomized trial among low-birth-weight children receiving bacillus Calmette-Guerin vaccination at first health center contact. Pediatr Infect Dis J 31: 306-308.

25. Hersh EM, Gutterman JU, Mavligit GM (1977) BCG as adjuvant immunotherapy for neoplasia. Annu Rev Med 28: 489-515.

26. Ifrim DC, Quintin J, Joosten LA, Jacobs C, Jansen T, et al. (2014) Trained immunity or tolerance: opposing functional programs induced in human monocytes after engagement of various pattern recognition receptors. Clin Vaccine Immunol 21: 534-545.

27. Rafnar T, Vermeulen SH, Sulem P, Thorleifsson G, Aben KK, et al. (2011) European genome-wide association study identifies SLC14A1 as a new urinary bladder cancer susceptibility gene. Hum Mol Genet 20: 4268-4281.

28. Songane M, Kleinnijenhuis J, Alisjahbana B, Sahiratmadja E, Parwati I, et al. (2012) Polymorphisms in autophagy genes and susceptibility to tuberculosis. PLoS One 7: e41618.

29. Smeekens SP, Ng A, Kumar V, Johnson MD, Plantinga TS, et al. (2013) Functional genomics identifies type I interferon pathway as central for host defense against Candida albicans. Nat Commun 4: 1342.

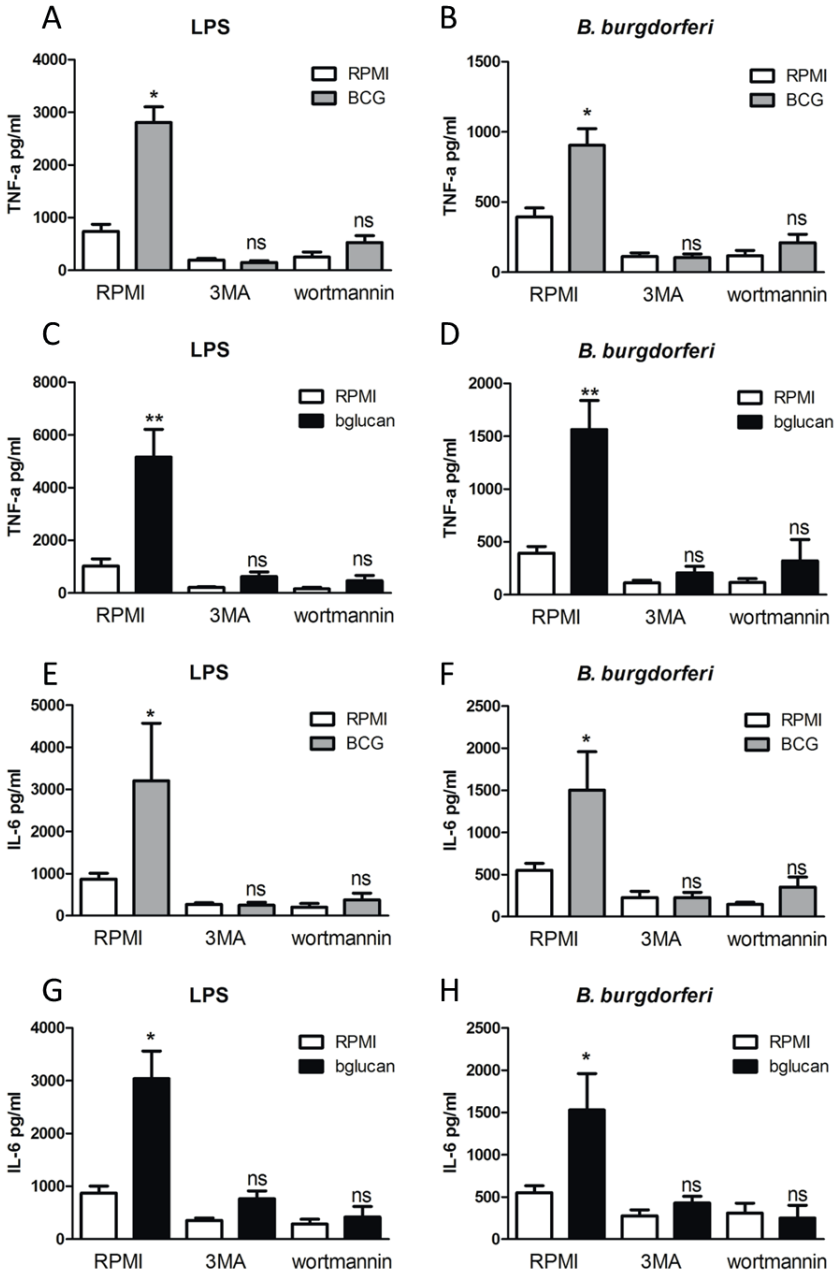


Figure S1. Role of autophagy for the training of monocytes. BCG (A-B, E-F) or β-glucan (C-D, G-H) training in vitro in the presence or absence of 3MA or wortmannin using freshly isolated human monocytes and different stimuli for restimulation (LPS, B. burgdorferi). TNF-α (A-D) and IL-6 (E-H) were assessed by ELISA in the supernatants. *P<0.05, **P<0.01.

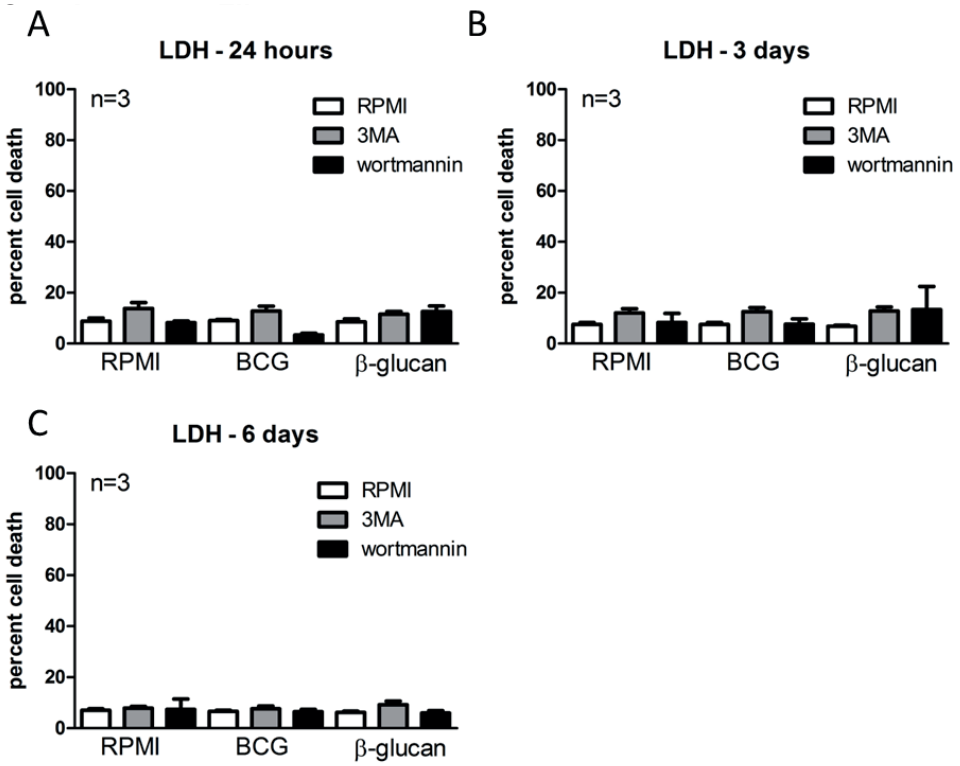
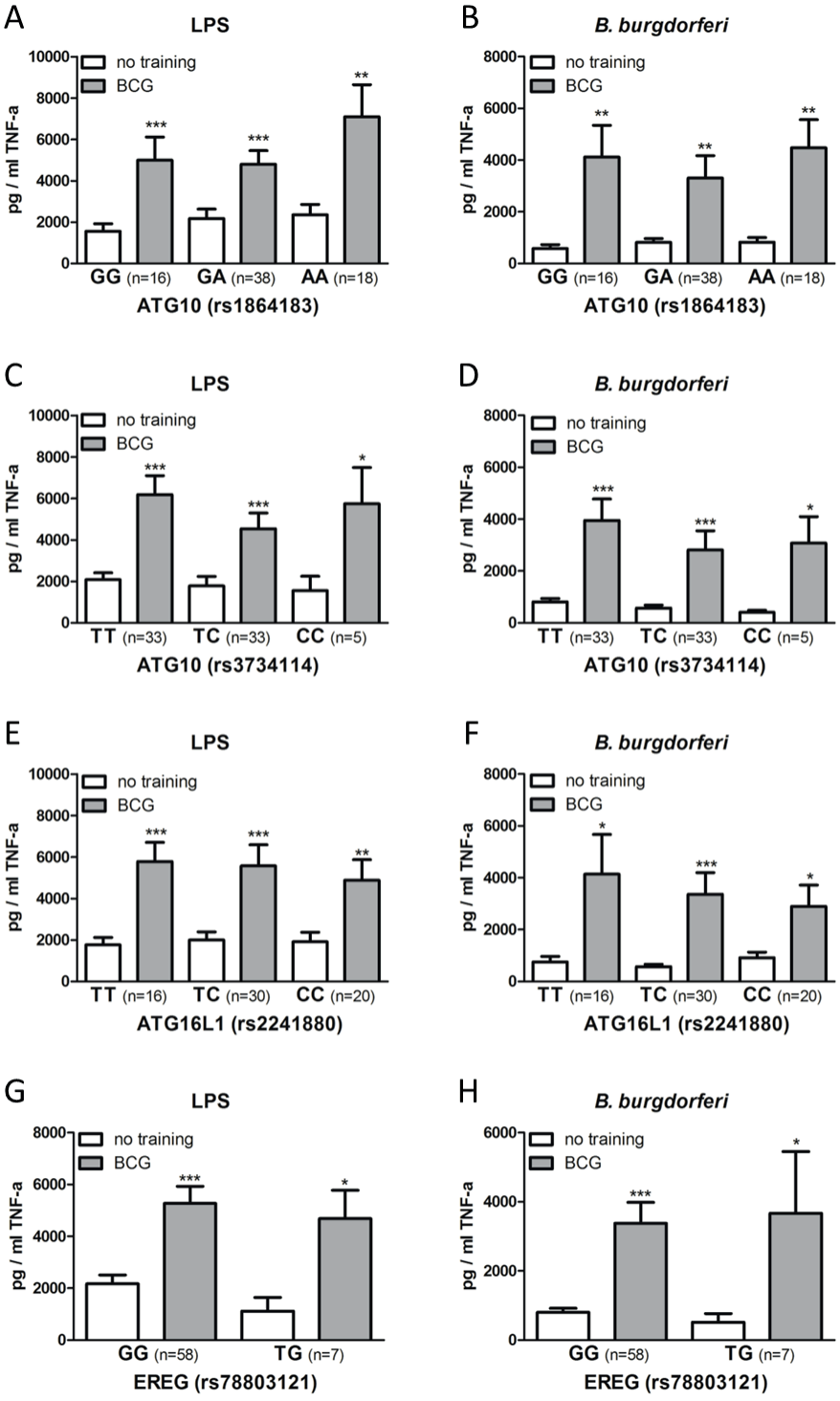


Figure S2. Viability of monocytes after chemical blocking of autophagy for 24h. BCG or β-glucan training in vitro in the presence or absence of 3MA or wortmannin using freshly isolated human monocytes. Cell viability tested by CytoTox 96 NonRadioactive Cytotoxicity Assay after 24h (A), 3 days (B) and 6 days (C).


A
LPS
pg / ml TNF-a
no training
BCG
GG (n=16)
GA (n=38)
AA (n=18)
ATG10 (rs1864183)
B
B. burgdorferi
C
LPS
TT (n=33)
TC (n=33)
CC (n=5)
ATG10 (rs3734114)
D
E
LPS
TT (n=16)
TC (n=30)
CC (n=20)
ATG16L1 (rs2241880)
F
G
LPS
GG (n=58)
TG (n=7)
EREG (rs78803121)
H

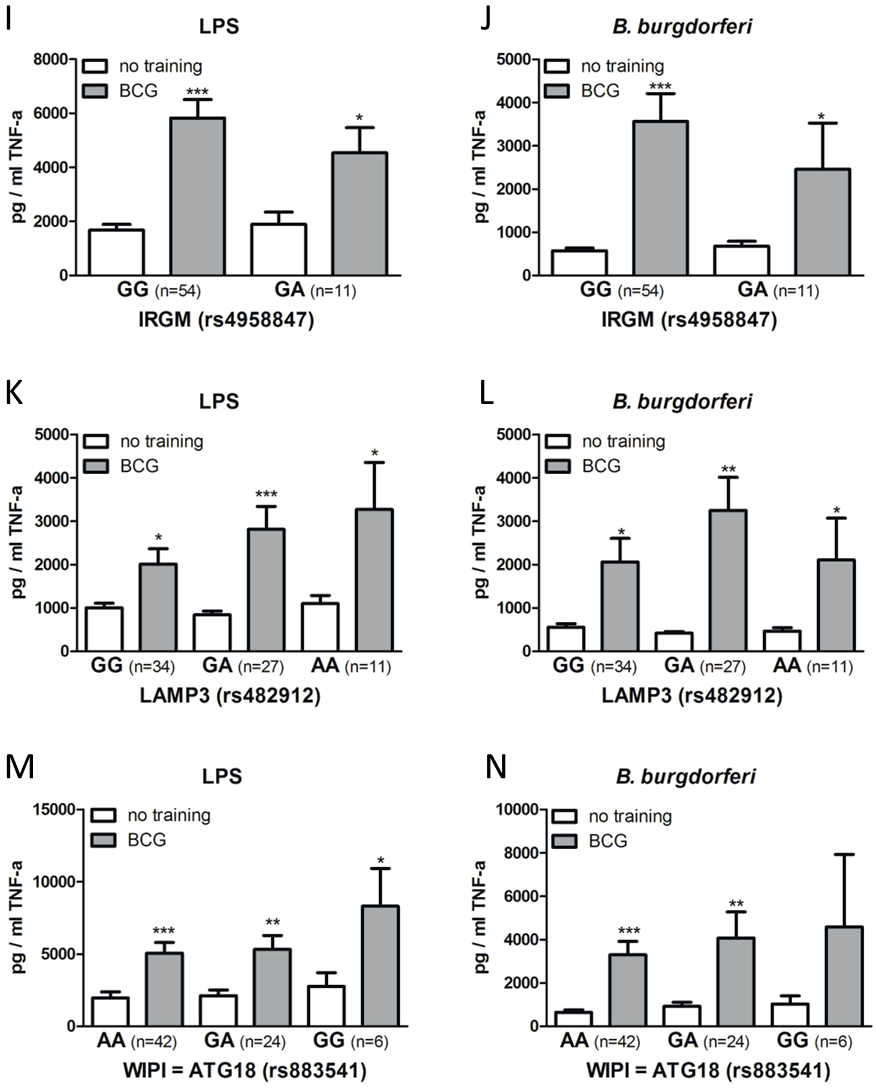


Figure S3. Polymorphisms in ATG10, ATG16L1, EREG, IRGM, LAMP3 and ATG18 do not diminish the training capacity of human monocytes. Blood was collected from volunteers and genotyped for ATG10 rs1864183 and rs3734114 (A-D), ATG16L1 rs2241880 (E-F), EREG rs78803121 (G-H), IRGM rs4958847 (I-J), LAMP3 rs482912 (K-L) and ATG18 rs8835411 (M-N). Human monocytes were trained with BCG for 24 h, washed and incubated in RPMI (10% human serum) for 6 d, after which they were restimulated for 24h with a second stimulus (LPS or Bb). Proinflammatory cytokine production (TNF-α) was assessed by ELISA in the supernatants.

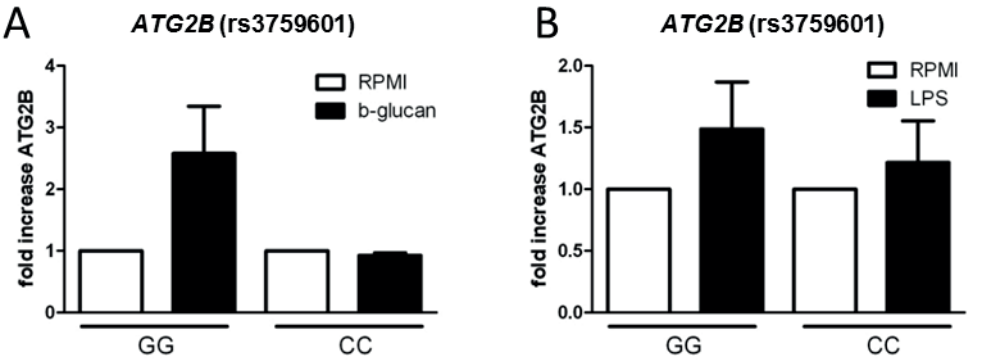


Figure S4. SNP in ATG2B affects its expression after training but not stimulation. Human monocytes carrying different genotypes for SNP rs3759601 were trained with β-glucan [A] or stimulated with LPS [B] for 4h. Expression of ATG2B was assessed by qPCR.

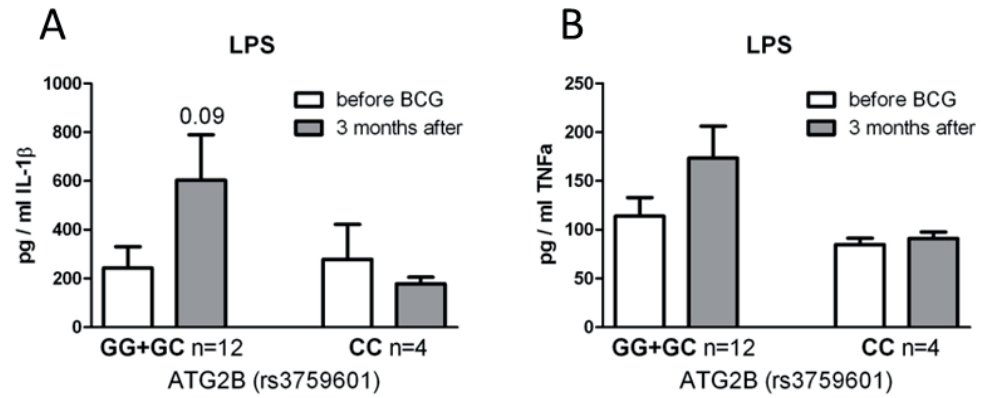


Figure S5. SNP in ATG2B affects the efficacy of in vivo BCG-induced trained immunity. Monocytes isolated before and 3 months after vaccination of 16 naïve (nonexposed) volunteers were stimulated in vitro with LPS. Proinflammatory cytokine production (IL-1β [A], TNF-α [B]) was assessed by ELISA in the supernatants.

Chapter 7

Summary and general conclusion

Lyme disease, caused by spirochetes of the *Borrelia burgdorferi* sensu lato complex, is the most common vector-born disease in the northern hemisphere. The disease was diagnosed as a separate condition for the first time in Old Lyme, Connecticut in 1975 followed by the description of the bacterium involved in 1981 by Willy Burgdorfer [1, 2]. The knowledge about *B. burgdorferi* has increased significantly in the last three decades. The transmission of the bacterium takes place after the attachment of an infected *Ixodes* tick. After *B. burgdorferi* is injected into the skin by an infected tick, the host's immune system induces a rapid inflammatory response [3].

As an introduction to this thesis, we explored the signaling pathways involved in the recognition of *B. burgdorferi* by immune cells and their effect on the induction of cytokines in **Chapter 2**. Using several knockout mice, we were able to investigate the role of TLR-, NLR- and IL-1 signaling during the stimulation with *B. burgdorferi*. Murine macrophages lacking the IL-1 receptor expressed an abrogated cytokine response to the spirochete illustrating the important role of IL-1 during murine Lyme arthritis. In addition, we reported a critical role of the TLR2-Myd88 pathway activating Caspase-1 which is responsible for the cleavage of inactive pro-IL-1β into its active form. Although NOD2 has been shown to play an important role on the induction of pro-inflammatory cytokines after *Borrelia* recognition in humans, NOD1 and NOD2 seem to be redundant in the investigated murine model.

NOD2 is a receptor which has been associated with the induction of the intracellular degradation process called autophagy [4]. Since autophagy has been linked to the modulation of cytokine production in several diseases [5], we investigated its role on *Borrelia*-induced cytokines in **Chapter 3**. First, we demonstrated the induction of autophagy by the spirochete. In addition, by stimulating human peripheral blood mononuclear cells (PBMCs), we proved that inhibition of autophagy increased pro-inflammatory innate cytokines after stimulation with *Borrelia* bacteria. Since previous studies have shown that autophagy modulated the production of T-cell derived cytokines [6] and elevated levels of IL-17 have been found in patients with confirmed neuroborreliosis [7], we investigated the role of autophagy on *Borrelia*-induced adaptive cytokines in **Chapter 4**. Higher levels of IL-17, IL-22 and IFN-γ protein were found in autophagy-blocked PBMCs stimulated with the *Borrelia*-spirochete, demonstrating a regulatory link between autophagy and T-cell cytokine production in response to *B. burgdorferi* stimulation.

Assessment of clinical symptoms and immune characteristics of *Borrelia*-infected patients illustrates a large diversity in clinical symptoms which have been associated to genetic variations in these patients leading to differences in clinical outcome [8]. In **Chapter 5**, we investigated the role of genetic variations in autophagy genes and their effect on *Borrelia*-induced cytokine responses. Significant up- and downregulation of several autophagy-related genes as ATG7, ATG9, and ATG16L1 was found in PBMCs exposed to *B. burgdorferi*. Different expression levels of those genes could also be found comparing Lyme disease patients to healthy controls indicating a relevant role for those genes in the pathogenesis of the disease. In addition, we looked into the effect of differentially expressed autophagy-related genes on the production of inflammatory cytokines. Single nucleotide polymorphisms (SNPs) in autophagy genes modulating the expression of inflammatory cytokines could be one explanation for the different disease outcome.

In recent years, emerging evidence has shown that after infection or vaccination, innate immune cells display long-term changes in their functional programs. These changes lead to increased production of inflammatory mediators and enhanced capacity to eliminate infection [9]. In **Chapter 6** the role of autophagy in this process, called trained immunity, has been investigated. Polymorphisms in autophagy genes such as ATG2B and ATG5 control trained immunity in vitro and in vivo. *Borrelia*-stimulated monocytes from volunteers carrying an ATG polymorphism produced the same amount of cytokines before and after vaccination in contrast to wildtype monocytes which produced elevated levels of IL-1β three months post-vaccination. A vaccination with BCG could therefore have a protective role for future infections with *B. burgdorferi* in individuals with intact autophagy.

General conclusion and future perspectives

The incidence of Lyme disease has increased in the past few years, pointing to the urgency of new developments in treatment options. The Infectious Diseases Society of America published guidelines for the treatment of Lyme disease including oral antibiotics as a first line therapy and intravenously-applied antibiotics for patients with more severe symptoms [10]. Nevertheless, in approximately 10% of the patients symptoms may persist [11]. The reason for persistent symptoms in some patients after treatment for Lyme disease is not known. Theories include residual damage to tissue, slow resolution of the inflammatory

state, or a form of cytokine-induced sickness behavior due to previously high levels of circulating cytokines [12].

In this thesis, we studied the effect of autophagy on Lyme disease in order to provide novel strategies for the treatment of the disease. We have demonstrated the induction of autophagy by *Borrelia burgdorferi* and its regulatory function on the production of *Borrelia*-induced cytokines. It is tempting to speculate that inhibition of autophagy could be beneficial during the early phase of Lyme disease leading to a higher concentration of innate cytokines and therefore a possibly improved clearance of the pathogen. However, because Lyme disease occurs in different stadia, enhanced IL-1β production by autophagy modulation in disseminated Lyme disease might be detrimental for the disease activity. Several studies indicated a link between a high concentration of IL-1β and pathogenic Th17 cells leading to increased joint damage in rheumatoid arthritis [13]. Therefore, the induction of autophagy in the disseminated state of Lyme disease could be favorable for Lyme patients through a decreased IL-1β production and inhibition of pathogenic Th17 cells. In the future, it might be possible to test patients for their intrinsic autophagy potential and adapt treatment options according the stadium of the disease and the status of their autophagy capability. Our findings underline the important role of autophagy in the pathogenesis of Lyme disease and suggest that modulation of autophagy could be a novel therapeutic strategy in that disease.

In the last chapter of this thesis, we studied the effect of autophagy on trained immunity induced by BCG. Emerging evidence has shown that after infection or vaccination, innate immune cells display long-term changes in their functional programs. These changes lead to increased production of inflammatory mediators and enhanced capacity to eliminate infection.

We discovered that autophagy is a central event modulating trained immunity induced by BCG which opens new possibilities for improvement of future BCG-based vaccines to be used against infections such as Lyme Disease. The addition of an autophagy-inducing agent to the vaccine could lead to improved trained immunity, and therefore an enhanced therapeutic potential.

References

1. Steere, A.C., et al., *Lyme arthritis: an epidemic of oligoarticular arthritis in children and adults in three connecticut communities.* Arthritis Rheum, 1977. **20**(1): p. 7-17.

2. Burgdorfer, W., et al., *Lyme disease-a tick-borne spirochetosis?* Science, 1982. **216**(4552): p. 1317-9.

3. Oosting, M., et al., *Innate immunity networks during infection with Borrelia burgdorferi.* Crit Rev Microbiol, 2016. **42**(2): p. 233-44.

4. Homer, C.R., et al., *A dual role for receptor-interacting protein kinase 2 (RIP2) kinase activity in nucleotide-binding oligomerization domain 2 (NOD2)-dependent autophagy.* J Biol Chem, 2012. **287**(30): p. 25565-76.

5. Harris, J., et al., *Autophagy controls IL-1beta secretion by targeting pro-IL-1beta for degradation.* J Biol Chem, 2011. **286**(11): p. 9587-97.

6. Peral de Castro, C., et al., *Autophagy regulates IL-23 secretion and innate T cell responses through effects on IL-1 secretion.* J Immunol, 2012. **189**(8): p. 4144-53.

7. Henningsson, A.J., et al., *Indications of Th1 and Th17 responses in cerebrospinal fluid from patients with Lyme neuroborreliosis: a large retrospective study.* J Neuroinflammation, 2011. **8**: p. 36.

8. Netea, M.G., F.L. van de Veerdonk, and J.W. van der Meer, *Primary immunodeficiencies of pattern recognition receptors.* J Intern Med, 2012. **272**(6): p. 517-27.

9. Netea, M.G., et al., *Trained immunity: A program of innate immune memory in health and disease.* Science, 2016. **352**(6284): p. aaf1098.

10. Wormser, G.P., et al., *The clinical assessment, treatment, and prevention of lyme disease, human granulocytic anaplasmosis, and babesiosis: clinical practice guidelines by the Infectious Diseases Society of America.* Clin Infect Dis, 2006. **43**(9): p. 1089-134.

11. Steere, A.C., J. Coburn, and L. Glickstein, *The emergence of Lyme disease.* J Clin Invest, 2004. **113**(8): p. 1093-101.

12. Bockenstedt, L.K. and G.P. Wormser, *Review: unraveling Lyme disease.* Arthritis Rheumatol, 2014. **66**(9): p. 2313-23.

13. Geboes, L., et al., *Proinflammatory role of the Th17 cytokine interleukin-22 in collagen-induced arthritis in C57BL/6 mice.* Arthritis Rheum, 2009. **60**(2): p. 390-5.

Chapter 8

Nederlandse samenvatting en conclusie

De ziekte van Lyme, veroorzaakt door bacteriën van het *Borrelia burgdorferi* sensu lato complex, is de meest verspreide vector-veroorzaakte ziekte in de noordelijke hemisfeer. De ziekte werd voor het eerst gediagnosticeerd in Old Lyme, Connecticut, in het jaar 1975 gevolgd door de beschrijving van de bacteriën in het jaar 1981 door Willy Burgdorfer. De kennis over *Borrelia* is toegenomen in de afgelopen drie decennia. Besmetting met deze bacterie vindt plaats nadat een geïnfecteerde teek meer dan 24 uur heeft vastgezeten op de huid. Nadat de pathogeen in de gastheer terecht komt, induceert het afweersysteem zeer snel een immuunrespons tegen *Borrelia*.

Als inleiding van dit proefschrift hebben wij de signaalwegen die betrokken zijn bij de herkenning van *Borrelia* door immuuncellen onderzocht **(Hoofdstuk 2)**. We hebben de rol van Toll-like receptors (TLR), NOD-like receptors (NLR), en IL-1 signalen bestudeerd in een muismodel met Lyme artritis, door gebruik te maken van verschillende knock-out muizen. Wanneer macrofagen van muizen, die IL-1 receptor moleculen mistten, werden gestimuleerd met *Borrelia* spirocheten, werd een verlaging van de cytokineproductie waargenomen. We rapporteerden ook een belangrijke rol voor de TLR2-Myd88 cascade voor de activering van caspase-1, een molecuul dat verantwoordelijk is voor het splitsen van pro-IL-1β in zijn actieve vorm. Hoewel NOD2 een belangrijke rol speelt tijdens de inductie van humaan pro-inflammatoire cytokines na de herkenning van *Borrelia* bacterie, schijnen NOD1 en NOD2 overbodig te zijn in ons muismodel.

NOD2 is een receptor die onder andere met de inductie van intracellulaire degradatieprocessen, ook autofagie genoemd, wordt geassocieerd. Omdat autofagie met de modulatie van cytokinenproductie in verschillende ziektes wordt verbonden, hebben wij de rol van autofagie op Borrelia-geïnduceerde cytokinenproductie onderzocht **(Hoofdstuk 3)**. Ten eerste hebben wij de inductie van autofagie door *Borrelia* laten zien. Bovendien konden wij door de stimulatie van humane perifere bloed mononucleaire cellen (PBMCs) aantonen dat het remmen van autofagie de *Borrelia*-afhankelijke cytokinenproductie verhoogd. Eerdere studies hebben laten zien dat autofagie de productie van T-cel afhankelijke cytokines kan moduleren en verhoogde hoeveelheden van IL-17 werden in patiënten met neuroborreliose gevonden. Dat was voor ons de reden om de rol van autofagie op Borrelia-geïnduceerde adaptieve cytokines te onderzoeken **(Hoofdstuk 4)**. Hoge levels van IL-17, IL-22 en IFN-γ konden wij aantonen in *Borrelia*-gestimuleerde PBMCs waarbij autofagie geblokkeerd was. Dit laat een regulatorische link zien tussen autofagie en de productie van T-cel cytokines na stimulatie van afweercellen met deze bacteriën.

Het vastleggen van klinische symptomen en eigenschappen van de afweerreactie van patiënten die met *Borrelia*-bacteriën geïnfecteerd zijn laat een grote diversiteit zien van klinische verschijnselen die geassocieerd zijn met genetische variatie. Deze diversiteit leidt ook tot verschillen in succes van de behandeling. In **Hoofdstuk 5** hebben wij de rol van genetische variatie van autofagie genen en het effect op *Borrelia*-geïnduceerde cytokinenproductie onderzocht. Significante veranderingen van meerdere autofagie-genen zoals ATG7, ATG9, en ATG16L1 werden gevonden in PBMCs die met *Borrelia* werden gestimuleerd. Verschillende expressie hoeveelheden van deze genen werden gevonden als patiënten monsters met monsters van gezonde vrijwilligers werden vergeleken. Dit resultaat laat een relevante rol van deze genen in de pathogenese van de ziekte van Lyme zien. Het effect van polymorfismen in autofagie genen op de expressie van inflammatoire cytokines zou een mogelijke verklaring voor verschillen in ziektebeeld kunnen zijn.

In recente jaren hebben verschillende studies aangetoond dat vroege afweercellen langdurige veranderingen in hun afweerfuncties hebben door een infectie of een vaccinatie. Deze veranderingen leiden tot een verhoogde productie van inflammatoire mediatoren en een verhoogde capaciteit om een infectie te stoppen. In **Hoofdstuk 6** werd de rol van autofagie op dit proces, trained immunity genoemd, onderzocht. Polymorfismen in autofagiegenen zoals ATG2B en ATG5 controleren trained immunity in vitro en in vivo. *Borrelia*-gestimuleerde monocyten van vrijwilligers met een ATG polymorfisme produceren dezelfde hoeveelheid cytokines voor en na een vaccinatie in tegenstelling tot wildtype monocyten die een hogere hoeveelheid IL-1β produceren drie maanden na de vaccinatie. Een vaccinatie met BCG zou daarom een beschermende rol voor een latere infectie met *Borrelia* kunnen hebben in mensen met een intact autofagie-systeem.

Algemene conclusies en toekomstperspectieven

De incidentie van de ziekte van Lyme is toegenomen in de afgelopen jaren en wijst op de urgentie van nieuwe ontwikkelingen van behandelingen. De ‘Infectious Diseases Society of America’ heeft nieuwe richtlijnen van behandelingen tegen de ziekte van Lyme gepubliceerd. Orale antibiotica worden na de eerste symptomen voorgeschreven. Voor patiënten met ernstigere verschijnselen worden antibiotica voorgeschreven die in het bloed worden toegediend. Desondanks houdt 10% van de patiënten klachten na behandeling. De oorzaak van aanhoudende verschijnselen van sommige patiënten na de behandeling is niet bekend. Verschillende theorieën beweren dat dit verschillende

oorzaken kan hebben, waaronder rest-schade aan weefsels, een langzame verdwijning van het ontstekingsproces of een vorm van ziekte geïnduceerd door aanwezigheid van hoge concentraties van cytokines tijdens de ontsteking.

In dit proefschrift hebben wij het effect van autofagie op de ziekte van Lyme onderzocht om nieuwe strategieën voor de behandeling van deze ziekte te vinden. Wij konden inductie van autofagie door *Borrelia* laten zien, maar ook de regulatorische functie van autofagie op de productie van *Borrelia*-geïnduceerde cytokines. Wij speculeren dat de remming van autofagie belangrijk zou kunnen zijn in de eerste fase van de ziekte omdat een hogere concentratie van vroege cytokines door de remming van autofagie word geproduceerd, wat de eliminatie van het pathogeen zou bespoedigen. De ziekte van Lyme heeft verschillende stadia; verhoogde productie van IL-1β zou nadelig kunnen zijn in een later stadium van de ziekte. Inductie van autofagie zou voordelig kunnen zijn voor Lyme patiënten in een later stadium van de ziekte omdat een verlaagde productie van IL-1β een verlaagde hoeveelheid Th17 cellen ten gevolge heeft. In de toekomst zou het mogelijk kunnen zijn om patiënten op de werking van autofagie te kunnen testen en behandelingen aan te passen op het stadium van de ziekte en de staat van autofagie. Onze resulaten onderschrijven de belangrijke rol van autofagie tijdens te pathogenese van de ziekte van Lyme en suggereerd dat modulatie van autofagie een potentiële nieuwe behandelingsoptie voor deze ziekte zou kunnen zijn.

In het laatste hoofdstuk van dit proefschrift hebben wij het effect van autofagie op “trained immunity” onderzocht. Nieuw onderzoek heeft laten zien dat vroege immuuncellen na infectie of vaccinatie langdurige veranderingen oplopen. Deze veranderingen leiden tot een verhoogde productie van inflammatoire mediatoren en een verhoogde capaciteit om infecties te stoppen.

Wij hebben ontdekt dat autofagie een centrale rol speelt bij de modulatie van trained immunity geïnduceerd door BCG. Dit biedt nieuwe mogelijkheden voor verbeteringen in toekomstige BCG-gebaseerde vaccinaties die gebruikt kunnen worden tegen infecties zoals de ziekte van Lyme. Het toevoegen van een autofagie-inducerende stof zou de training van de afweercellen na de vaccinatie kunnen bevorderen, en op deze manier de effectiviteit van de vaccinatie te verhogen.

References

1. Steere, A.C., et al., *Lyme arthritis: an epidemic of oligoarticular arthritis in children and adults in three connecticut communities.* Arthritis Rheum, 1977. **20**(1): p. 7-17.

2. Burgdorfer, W., et al., *Lyme disease-a tick-borne spirochetosis?* Science, 1982. **216**(4552): p. 1317-9.

3. Oosting, M., et al., *Innate immunity networks during infection with Borrelia burgdorferi.* Crit Rev Microbiol, 2016. **42**(2): p. 233-44.

4. Homer, C.R., et al., *A dual role for receptor-interacting protein kinase 2 (RIP2) kinase activity in nucleotide-binding oligomerization domain 2 (NOD2)-dependent autophagy.* J Biol Chem, 2012. **287**(30): p. 25565-76.

5. Harris, J., et al., *Autophagy controls IL-1beta secretion by targeting pro-IL-1beta for degradation.* J Biol Chem, 2011. **286**(11): p. 9587-97.

6. Peral de Castro, C., et al., *Autophagy regulates IL-23 secretion and innate T cell responses through effects on IL-1 secretion.* J Immunol, 2012. **189**(8): p. 4144-53.

7. Henningsson, A.J., et al., *Indications of Th1 and Th17 responses in cerebrospinal fluid from patients with Lyme neuroborreliosis: a large retrospective study.* J Neuroinflammation, 2011. **8**: p. 36.

8. Netea, M.G., F.L. van de Veerdonk, and J.W. van der Meer, *Primary immunodeficiencies of pattern recognition receptors.* J Intern Med, 2012. **272**(6): p. 517-27.

9. Netea, M.G., et al., *Trained immunity: A program of innate immune memory in health and disease.* Science, 2016. **352**(6284): p. aaf1098.

10. Wormser, G.P., et al., *The clinical assessment, treatment, and prevention of lyme disease, human granulocytic anaplasmosis, and babesiosis: clinical practice guidelines by the Infectious Diseases Society of America.* Clin Infect Dis, 2006. **43**(9): p. 1089-134.

11. Steere, A.C., J. Coburn, and L. Glickstein, *The emergence of Lyme disease.* J Clin Invest, 2004. **113**(8): p. 1093-101.

12. Bockenstedt, L.K. and G.P. Wormser, *Review: unraveling Lyme disease.* Arthritis Rheumatol, 2014. **66**(9): p. 2313-23.

13. Geboes, L., et al., *Proinflammatory role of the Th17 cytokine interleukin-22 in collagen-induced arthritis in C57BL/6 mice.* Arthritis Rheum, 2009. **60**(2): p. 390-5.

Chapter 9

List of publications
Curriculum vitae
Acknowledgments

List of publications

1. **Buffen K**, Oosting M, Li Y, Kanneganti TD, Netea MG, Joosten LA. *Autophagy suppresses host adaptive immune responses toward Borrelia burgdorferi.* **J Leukoc Biol. 2016** Sep; 100(3):589-98.

2. Oosting M, **Buffen K**, van der Meer JW, Netea MG, Joosten LA. *Innate immunity networks during infection with Borrelia burgdorferi.* **Crit Rev Microbiol. 2016**; 42(2):233-44.

3. Oosting M, **Buffen K**, Cheng SC, Verschueren IC, Koentgen F, van de Veerdonk FL, Netea MG, Joosten LA. *Borrelia-induced cytokine production is mediated by spleen tyrosine kinase (Syk) but is Dectin-1 and Dectin-2 independent.* **Cytokine. 2015** Dec; 76(2):465-72.

4. **Buffen K**, Oosting M, Quintin J, Ng A, Kleinnijenhuis J, Kumar V, van de Vosse E, Wijmenga C, van Crevel R, Oosterwijk E, Grotenhuis AJ, Vermeulen SH, Kiemeney LA, van de Veerdonk FL, Chamilos G, Xavier RJ, van der Meer JW, Netea MG, Joosten LA. *Autophagy controls BCG-induced trained immunity and the response to intravesical BCG therapy for bladder cancer.* **PLoS Pathog. 2014** Oct 30; 10(10).

5. Smeekens SP, Malireddi RK, Plantinga TS, **Buffen K**, Oosting M, Joosten LA, Kullberg BJ, Perfect JR, Scott WK, van de Veerdonk FL, Xavier RJ, van de Vosse E, Kanneganti TD, Johnson MD, Netea MG. *Autophagy is redundant for the host defense against systemic Candida albicans infections.* **Eur J Clin Microbiol Infect Dis. 2014** May; 33(5):711-22.

6. **Buffen K**, Oosting M, Mennens S, Anand PK, Plantinga TS, Sturm P, van de Veerdonk FL, van der Meer JW, Xavier RJ, Kanneganti TD, Netea MG, Joosten LA. *Autophagy modulates Borrelia burgdorferi-induced production of interleukin-1*β *(IL-1*β*).* **J Biol Chem. 2013** Mar 22; 288(12):8658-66.

7. Oosting M, **Buffen K**, Malireddi SR, Sturm P, Verschueren I, Koenders MI, van de Veerdonk FL, van der Meer JW, Netea MG, Kanneganti TD, Joosten LA. *Murine Borrelia arthritis is highly dependent on ASC and caspase-1, but independent of NLRP3.* **Arthritis Res Ther. 2012** Nov 13; 14(6).

Curriculum Vitae

Kathrin Erler (née Buffen) was born in Kevelaer, Germany on October 23rd, 1986. She attended high school at the Lise-Meitner-Gymnasium in Geldern (Germany) and received her diploma in 2006. During her high school years, Kathrin spent some months at the DC Everest High School in Schofield, Wisconsin. In 2009 she obtained her Bachelor Degree in Medical Biology from Radboud University Nijmegen. Kathrin performed the last year of her Bachelor's program at the University of Edinburgh, Scotland. Between 2009 and 2011 she enrolled in a Master course in Medical Biology at Radboud University, Nijmegen. She performed two scientific internships at the department of Experimental Rheumatology (NCMLS) in Nijmegen, Netherlands and at the T Cell Biology lab (Centenary Institute of Cancer Medicine and Cell Biology) in Sydney, Australia.

In 2011 she started her Ph.D. research at the Department of Internal Medicine, Radboudumc, under supervision of prof. dr. L.A.B. Joosten and prof. dr. M.G. Netea. The aim of the Ph.D. thesis was to unravel the role of autophagy in host defence against *Borrelia burgdorferi.* During her Ph.D. studies, she presented her research at different international conferences, e.g. ICLB (International Conference on Lyme Borreliosis) in Boston, USA and Keystone Symposia on Autophagy, Inflammation and Immunity in Montreal, Canada.

In 2013, Kathrin moved to Essen, Germany, to study medicine at the University of Duisburg-Essen. She finished her Ph.D. research next to her studies and started to work under the supervision of prof. dr. M. Gunzer at the Institute for Experimental Immunology and Imaging in Essen, Germany in 2015. She investigates the distribution of Aspergillus fumigatus in different mouse models.

In September 2016 Kathrin married Lukas Erler.